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REMARKS

Claims 1-33 are pending in the application. Claims 1-19 and 21-33 have been withdrawn and are hereby cancelled without prejudice: applicants expressly reserve the right to re-file claims 1-19 and 21-33 in a divisional application. Claim 20 has been finally rejected as being anticipated by *Holmvall*, *Pfaff*, or *Kern* and as being obvious over *Qu*.

Applicants respectfully request that the Examiner enter the instant amendments of claim 20 as the amendments address all of the outstanding grounds for rejection, do not raise issues of new matter, and do not present new issues requiring further consideration or search.

The proposed amendments to claim 20 clarify that the crystallographic coordinates of the I-domain of the $\alpha 1$ chain of the $\alpha 1\beta 1$ integrin, or a complex comprising an $\alpha 1\beta 1$ integrin, or homologs thereof, are employed computationally or experimentally to perform a fitting operation between the chemical entity and the $\alpha 1\beta 1$ integrin I-domain or complex thereof, thereby obtaining data related to the association. *Holmvall*, *Pfaff*, *Kern*, and *Qu* do not describe the use of crystallographic coordinates of the I-domain of the $\alpha 1$ chain in the method of claim 20 as specified in the proposed amendments. (The complete texts of *Holmvall*, *Pfaff*, *Kern*, and *Qu* are attached for the Examiner's consideration.)

Holmvall, *Pfaff*, and *Kern* do not disclose the use of crystallographic data to evaluate interactions involving a chemical entity and a ligand. *Holmvall* used affinity chromatography to assess the binding of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins to collagen type II. Similarly, *Pfaff* determined that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, when purified by collagen affinity chromatography, showed distinct binding to mouse tumor laminin-1. *Kern* investigated the interactions of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins and collagen IV using solid phase and inhibition assays.

Neither *Holmvall*, *Pfaff*, nor *Kern* disclosed a method for evaluating the ability of a chemical entity to associate with an I-domain of the $\alpha 1$ chain of the $\alpha 1\beta 1$ integrin, or a complex comprising an $\alpha 1\beta 1$ integrin, or homologs thereof, which used each and every limitation specified in the proposed amendments of claim 20. Accordingly, none of those

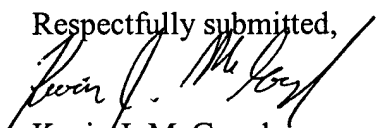
three references anticipate the method of claim 20 as specified in the proposed amendments.

Qu determined the high-resolution crystalline structure of the I-domain of CD11a with bound manganese ion and investigated the structural features of affinity modulation and ligand binding in this domain. *Qu* did not suggest, however, that the CD11a I-domain crystalline structure could be used in a fitting operation to assess the association between a chemical entity and an I-domain of CD11a. Nor is there any support for the Examiner's assertion that *Qu*'s mere identification of CD11a's I-domain crystalline structure rendered the method of claim 20 - which evaluates interactions involving a chemical entity and a different ligand - *prima facie* obvious.

The Examiner has not established any factual basis which provided those of ordinary skill in the art with a reason, suggestion, or motivation to use *Qu*'s crystalline structure of the I-domain of CD11a in a method which enables evaluation of the association between the chemical entity and an I-domain of the $\alpha 1$ chain of the $\alpha 1\beta 1$ integrin, or a complex comprising an $\alpha 1\beta 1$ integrin, or homologs thereof.

In light of all of the foregoing, Applicants respectfully submit that the instant amendments of claim 20 should be entered and that claim 20 should be passed to issue.

Respectfully submitted,



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Binding of purified collagen receptors ($\alpha 1\beta 1$, $\alpha 2\beta 1$) and RGD-dependent integrins to laminins and laminin fragments

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Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, when purified by collagen affinity chromatography, showed distinct binding to mouse tumor laminin-1, which has the chain composition $\alpha 1\beta 1\gamma 1$. The binding was, however, about 10-fold lower than to collagen IV. Only little ($\alpha 1\beta 1$) or no binding ($\alpha 2\beta 1$) was observed to two different laminin isoforms ($\alpha 2\beta 1\gamma 1$, $\alpha 2\beta 2\gamma 1$) from human placenta. Binding to laminin-1 was abolished by EDTA and could be specifically inhibited by antibodies to the respective integrin α subunit. These antibodies also inhibited cell adhesion to collagens. The binding of soluble integrins was weaker than that of immobilized integrins but could be enhanced by an activating anti($\beta 1$ integrin). No enhancement was observed for immobilized integrins. Studies with laminin-1 fragments demonstrated lack of binding to the major cell-adhesive fragment E8 from the long arm, fragments E3 and E4, involved in heparin-binding and self-assembly, respectively, and fragment P1, corresponding to the inner segments of the short arms. A larger short-arm fragment (E1XNd), which lacks the N-terminal $\beta 1$ chain domains V and VI, was as active as laminin. Together, these results, suggested the localization of the binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ to the N-terminal region of the laminin $\alpha 1$ chain. Fragment P1 but not intact laminin-1 bound to $\alpha V\beta 3$ integrin in an EDTA-sensitive and RGD-sensitive manner, underscoring previous data on the cryptic nature of the RGD site in laminin-1. Further analyses by surface plasmon resonance assays demonstrated a $K_D = 50$ nM for $\alpha 2\beta 1$ /laminin-1 binding and a $K_D = 450$ nM for $\alpha V\beta 3$ /fragment P1 binding and confirmed the anti- $\beta 1$ -mediated increase in affinity for $\alpha 2\beta 1$.

Laminin, which exists as various isoforms, has been established as a major cell-adhesive protein by its binding to several integrin and non-integrin receptors (Timpl, 1989; Mecham, 1991; Kramer et al., 1993). Integrin $\alpha 6\beta 1$ was originally identified as an abundant receptor for laminin-1 derived from the Engelbreth-Holm-Swarm (EHS) tumor (Sonnenberg et al., 1988), which has the chain composition $\alpha 1\beta 1\gamma 1$ (for nomenclature see Burgeson et al., 1994). The binding activity was localized to fragment E8, which corresponds to C-terminal domains from the distal end of the long arm of laminin (Aumailley et al., 1987; 1990a; Sonnenberg et al., 1990; 1991; Goodman et al., 1987; Hall et al., 1990). This interaction was shown to be essential for embryonic kidney development and is probably also important for many more biological activities of laminin (Ekblom, 1993). The E8-binding site is dependent on the conformation, with a major contribution made by the $\alpha 1$ chain (Deutzmann et al., 1990). Some muscle or melanoma cell lines seem to use the related integrin $\alpha 7\beta 1$ instead of $\alpha 6\beta 1$ for binding to E8 (von der Mark et al., 1991; Kramer et al., 1991). Further integrins implicated in laminin binding are $\alpha 1\beta 1$ (Hall et al., 1990; Forsberg et al., 1990; Tawil et al., 1990; Rossino et al., 1990; Kramer et al., 1990) and $\alpha 2\beta 1$ (Elices and Hemler, 1989; Languino et al., 1989; Kirchhofer et al., 1990), which are also the major receptors for various collagen types (Vandenberg et al., 1991; Gullberg et al., 1992; Pfaff et al., 1993;

Kern et al., 1993), and $\alpha 3\beta 1$ (Gehlsen et al., 1989; Sonnenberg et al., 1991). EHS tumor laminin-1 also possesses a cryptic RGD site which is recognized by $\beta 1$ and $\beta 3$ integrins, although their corresponding α chains have not been firmly identified (Aumailley et al., 1990b; 1991).

Superimposed on the diversity of integrin binding to EHS tumor laminin is the complexity generated by different laminin isoforms (Timpl and Brown, 1994). Cell attachment and neurite-stimulation studies with placental laminin variants ($\alpha 2\beta 1\gamma 1$, $\alpha 2\beta 2\gamma 1$) demonstrated mediation by $\beta 1$ integrin but no major role for the $\alpha 6$ subunit (Brown et al., 1994; Engvall et al., 1992). These observations were recently extended in studies with K562 cells transfected with either $\alpha 3$ or $\alpha 6$ integrin subunits (Delwel et al., 1993; 1994). The data indicated a strong binding of $\alpha 6\beta 1$ to EHS tumor laminin-1 and weaker but distinct binding to $\alpha 2$ chain-containing laminin isoforms and a further isoform referred to as laminin-5 ($\alpha 3\beta 3\gamma 2$). Integrin $\alpha 3\beta 1$ was a more specific receptor for laminin-5 and reacted only weakly or not at all with the other isoforms.

In the present study, we have investigated the binding of collagen receptors and several RGD-dependent integrins to three different laminin isoforms. This was carried out with purified integrins in order to allow quantitative analyses and to avoid complications arising from attachment studies with cells, which usually possess a complex repertoire of integrins. The data indicated similar and specific interactions of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins with laminin-1. The binding of RGD-dependent integrins was restricted to $\alpha V\beta 3$ and $\alpha IIb\beta 3$ with no activity observed for $\alpha 5\beta 1$.

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Abbreviation. EHS, Engelbreth-Holm-Swarm.

MATERIALS AND METHODS

Protein ligands, fragments and peptides

The preparation of the laminin-1-nidogen complex and collagen IV from the mouse EHS tumor and of several laminin-1 fragments (E3, E4, E8) followed established protocols (Timpl et al., 1987). Laminin-1 fragments E1XNd, P1X (Mann et al., 1988) and P1 (Aumailley et al., 1990b) were obtained as previously described. Samples of laminin-1-nidogen were, in addition, dialysed against either 0.1 M glycine/HCl, pH 1.9, or 0.1 M acetic acid, pH 3, incubated for 24 h at 25°C and subsequently dialysed against 0.05 M Tris/HCl, 0.1 M NaCl, pH 7.2. Laminin-2 and laminin-4 (chain compositions $\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 2\gamma 1$, respectively) were purified from neutral salt extracts of human placenta (Brown et al., 1994). A mixture of both isoforms in approximately equimolar proportions was prepared by omitting the MonoQ separation step. Recombinant mouse nidogen was obtained as previously described (Fox et al., 1991). Collagen IV and its fragment CB3 (Kern et al., 1993) were kindly supplied by J. Eble (Martinsried, Germany). Human plasma fibronectin (Behringwerke) and vitronectin (Yatohgo et al., 1988) were purified by heparin affinity chromatography. Human fibrinogen (Calbiochem) and synthetic GRGDS peptide (Bachem) were obtained from commercial sources.

Purification of Integrins

The sources of integrins were fresh human placentae or outdated human platelets. Single placentae were washed with water and extracted with 1 M NaCl in a neutral buffer containing protease inhibitors but no detergents (Brown et al., 1993; Pfaff et al., 1993). The residue was then homogenized in 500 ml buffer A (0.05 M Tris/HCl, pH 7.4, 0.15 M NaCl, 1 mM MnCl_2 , 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.5 mM phenylmethylsulfonyl fluoride and 1 $\mu\text{g/ml}$ each of aprotinin, antipain and leupeptin) containing either 0.5% Triton X-100 (Roth) or 25 mM octylglucoside (Senn Chemicals AG), stirred overnight at 4°C and the supernatant collected by centrifugation at 8000 $\times g$. The extraction step was repeated once and the combined supernatants centrifuged at 30000 $\times g$ and stored at -20°C. The washing and detergent extraction of platelets was performed as described by Vandenberg et al. (1991).

Affinity chromatography was carried out using 100–200 ml aliquots of the extracts on 5–10 ml columns of ligands which were coupled to CNBr-activated Sepharose (Pharmacia) following the manufacturer's instruction. The ligands used were the human collagen IV peptide CB3 (Kern et al., 1993) kindly provided by J. Eble (Martinsried, Germany), the synthetic peptide GRGDSPK (Pytela et al., 1987) kindly provided by Dr H. G. Zerwes (Sandoz), rat collagen I (Pfaff et al., 1993) and a 140-kDa elastase fragment of human fibronectin kindly provided by H. Richter (Martinsried, Germany), which is equivalent to the cell-binding chymotryptic 120-kDa fragment used previously (Pytela et al., 1987). Integrin $\alpha 1\beta 1$ was obtained on peptide CB3 from placental extracts in buffer A containing 0.5% Triton X-100 following a previously described elution protocol (Pfaff et al., 1993) which includes a final displacement step in buffer A containing 25 mM octylglucoside and 10 mM EDTA. Eluates were collected in 1.5 ml aliquots in vials containing 25 μl 1 M MgCl_2 . Similar protocols were followed to purify $\alpha 5\beta 1$ integrin on the fibronectin fragment and $\alpha V\beta 3$ integrin on GRGDSPK peptide using placental extracts with buffer A

and octylglucoside. Integrin $\alpha 2\beta 1$ was obtained from platelet extracts on collagen I (Vandenberg et al., 1991). Purified platelet integrin $\alpha \text{IIb}\beta 3$ was kindly supplied by J. Engel (Basel, Switzerland) and H. G. Zerwes (Basel, Switzerland) and purified by conventional chromatography (Müller et al., 1993).

Purified integrins in the EDTA eluates were examined by SDS/polyacrylamide electrophoresis (5–15% polyacrylamide gels), concentrated by centrifugation in Centricon-100 microconcentrators (Amicon) and, in the presence of 0.05% sodium azide, stored at 4°C. Protein concentrations were determined by a micro biconchic acid assay (Pierce) or by amino acid analysis after hydrolysis with 6 M HCl.

Sources of antibodies

Several polyclonal rabbit antibodies were used for detecting bound ligands in solid-phase assays and immunological assays. They included antisera against laminins and laminin fragments (Timpl et al., 1987; Brown et al., 1994), recombinant nidogen (Fox et al., 1991), fibronectin and various collagens which were prepared by standard protocols (Timpl, 1992). Rabbit antisera against human fibrinogen (DAKO GmbH) and human vitronectin (Biomol) were purchased. Further antisera against human $\alpha 2\beta 1$ integrin and the human $\beta 3$ subunit were donated by A. Kern and J. Calvete (both Martinsried, Germany), and those against human $\alpha V\beta 3$ integrin (Gibco BRL) and against the cytoplasmic domains of rat $\alpha 1$ and human $\alpha 2$, $\alpha 5$ and αV subunits (Chemicon Int.) were of commercial origin. Inhibitory and non-inhibitory monoclonal antibodies against integrins included anti- $\text{kb}1$ (AIB2) and anti- $\alpha 5$ (BIIG2, B1E5) supplied by C. H. Damsky (Hall et al., 1990), anti- $\beta 3$ (P97) from J. Calvete, and anti- $\alpha 1$ (1B3.1) from I. Bank (Briesewitz et al., 1993). An activating anti- $\beta 1$ (TS2/16; Arroyo et al., 1992) was supplied by F. Sanchez-Madrid (Madrid, Spain). Commercial sources (Dianova; Gibco BRL) were used to obtain anti- $\beta 1$ (P4C10), anti- $\alpha 2$ (Gi9), anti- $\alpha 4$ (P4G9), anti- $\alpha 5$ (SAM-1), anti- αV (AMF-7), anti- $\alpha \text{IIb}\beta 3$ (P2) and anti- $\alpha V\beta 3$ (P1F6). These antibodies were either available in purified form (Gi9, P2, P4G9, AMF-7, SAM-1), as hybridoma supernatants (P97, AIB2, BIIG2, B1E5, TS 2/16) or as ascites fluids (1B3.1, P4C10, P1F6) and, if necessary, their inhibitory activities were checked in cell adhesion assays.

Solid-phase binding assays with purified integrins

Binding between integrins and proteins using one ligand immobilized onto plastic wells was detected by antibodies against the soluble ligand. The antibodies were diluted to concentrations yielding an absorbance (490 nm) of 1.0–2.0 in regular ELISA. A previously described protocol (Pfaff et al., 1993) was used with some minor modifications. Coating of wells was carried out overnight at 4°C with ligands (2–30 $\mu\text{g/ml}$) dissolved in 0.05 M Tris/HCl, pH 7.4, 0.15 M NaCl (buffer B) containing, in addition, 2 mM MgCl_2 in the case of integrins. After blocking with 1% bovine serum albumin (Serva) in buffer B, all subsequent washing and binding steps (1–2 h, room temperature) were performed in buffer B containing 1 mM MnCl_2 , 1 mM MgCl_2 , 0.1 mM CaCl_2 and 0.04% Tween 20. Goat anti-rabbit IgG coupled to peroxidase (Bio-Rad) was used as second antibody. In inhibition assays, integrins were incubated (30 min) with monoclonal antibodies before adding the ligand. When required, activating antibody (TS2/16) was added after the incubation step. Peptide



Fig. 1. Electrophoresis of integrins purified from human placenta or platelets. The integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha V\beta 3$ and $\alpha IIb\beta 3$ were analysed in non-reduced (N) or reduced (R) form. Staining was with Coomassie brilliant blue. Positions of reduced calibrating proteins are indicated in the left margin.

GRGDS was added together with the ligand to immobilized integrins.

Surface plasmon resonance binding studies

A BIAcore™ biosensor system (Pharmacia Biosensor) together with the manufacturer's supplementary materials was used for real-time binding experiments (Fägerstam et al., 1992; Karlsson et al., 1991). The carboxymethylated dextran layer of Sensor chip CM5 was equilibrated in 10 mM HEPES, pH 7.5, 0.15 M NaCl, 3.4 mM EDTA, 0.05% (by vol.) BIAcore P20 surfactant, then activated with 0.05 M *N*-ethyl-*N*-(3-diethylaminopropyl)carbodiimide, 0.05 M *N*-hydroxysuccinimide dissolved in water (7 min). 35 μ l of the first ligand dissolved at 50 μ g/ml in 10 mM sodium acetate, pH 5.4, was injected at a flow rate of 5 μ l/min. Residual activated ester was then blocked with 50 μ l 0.1 M ethanolamine and washed with 20 μ l 10 mM EDTA in order to remove non-covalently bound ligand. Binding studies with the second ligand were performed in binding buffer, as used in the solid-phase binding assays (see above). Different concentrations of $\alpha 2\beta 1$ integrin (20–80 μ g/ml) and $\alpha V\beta 3$ integrin (25–140 μ g/ml) were used to determine association rate constants (k_{on}). Dissociation was monitored by replacing the integrin solution with binding buffer. For activation, integrin $\alpha 2\beta 1$ was mixed with 0.2 vol. antibody TS2/16. Kinetic rate constants were calculated with Microsoft Excel version 4.0 and Macros supplied by the manufacturer. Equilibrium dissociation constants (K_D) were calculated from the ratio k_{off}/k_{on} . After each binding analysis, full regeneration of the bound ligand was achieved with 20 mM EDTA in buffer B.

RESULTS

Characterization of purified integrins

Four human integrins were purified from placental or platelet extracts either by affinity chromatography on collagen IV or I ($\alpha 1\beta 1$, $\alpha 2\beta 1$), chromatography on a fibronectin fragment ($\alpha 5\beta 1$) or chromatography on synthetic GRGDSPK peptide ($\alpha V\beta 3$). A fifth human integrin ($\alpha IIb\beta 3$) was prepared from platelets by conventional chromatography without any affinity step (Müller et al., 1993). Analysis of these integrins by SDS/polyacrylamide electrophoresis and protein staining revealed two major bands of the expected sizes with a characteristic shift in mobility after reduction, and indicated a

purity >90% (Fig. 1). The integrins were further identified in immunoblots using monoclonal antibodies against the corresponding α and β subunits (not shown, but see Pfaff et al., 1993 for $\alpha 1\beta 1$). Immunoblots and ELISAs with immobilized integrins were also used to identify possible contaminations. This revealed minor $\alpha 5$ and αV bands in $\alpha 1\beta 1$ which, however, could only be visualized by protein staining after overloading the gel. Integrin $\alpha V\beta 3$ showed a trace contamination with the $\beta 5$ chain and integrin $\alpha 5\beta 1$ with the αV chain. In the case of $\alpha 5\beta 1$, inhibitory antibodies against the $\alpha 5$ subunit (SAM-1, P1B5 and BIIG2) blocked ligand binding to more than 90%, indicating that the contaminants do not contribute to the binding to a significant extent. Thus, the integrins appeared to be of sufficient purity for studying their interactions with laminins.

Binding of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins to different laminin isoforms

Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were originally identified as collagen receptors but have also been shown to interact with EHS tumor laminin-1, which has the chain composition $\alpha 1\beta 1\gamma 1$, in cell-attachment and affinity-chromatography studies (Hall et al., 1990; Forsberg et al., 1990; Elices and Hemler, 1989; Kramer et al., 1990). A comparison of collagen IV and laminin-1 binding to immobilized $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins confirmed their dual binding properties but showed an approximately 10-fold lower binding strength for laminin (Fig. 2). The binding of soluble integrins to immobilized laminin-1 was distinctly weaker. The interactions with laminin-1 depended on divalent cations and were abolished by EDTA (Fig. 3). The specificity of binding was also demonstrated by inhibition with specific anti- $\alpha 1$ and anti- $\alpha 2$ monoclonal antibodies (not shown, but see Fig. 4). EHS tumor laminin-1 is usually extracted as a stoichiometric non-covalent complex with nidogen. In order to exclude the participation of nidogen, we used a recombinant form and found only marginal binding to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. This binding seemed to be largely non-specific since it was only partially inhibited by EDTA (Fig. 3). Furthermore, we studied the exposure of laminin-1-nidogen complex to acidic conditions as used subsequently for the preparation of pepsin fragments P1 and P1X. Incubation at pH 1.9 or 3 (24 h, 25°C) caused no degradation of laminin and did not change the binding activity for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins (data not shown).

Two further laminin isoforms from human placenta, laminin-2 and laminin-4 (chain compositions $\alpha 2\beta 1\gamma 1$ and $\alpha 2\beta 2\gamma 1$, respectively) were used as a mixture in additional binding studies. No binding was observed to immobilized or soluble $\alpha 2\beta 1$ integrin, while a very weak but EDTA-sensitive reaction (absorbance of 0.1 at 20 μ g/ml) could be detected with soluble but not immobilized $\alpha 1\beta 1$ integrin. Stronger binding was observed with $\alpha IIb\beta 3$ integrin (absorbance of 0.25 at 20 μ g/ml) but was still 10–100-fold lower than the binding to fibronectin and fibrinogen. Studies with separated laminin-2 and laminin-4 demonstrated that both bind $\alpha IIb\beta 3$.

Mapping of integrin-binding sites on laminin-1

Several batches of defined laminin-1 fragments which cover most of the laminin structure (Timpi et al., 1987; Engel, 1993) were used to map the binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins (Fig. 3). The activity of fragment E1XNd was similar to that of intact laminin-1, even though a few batches with lower activity were also found. This fragment consists

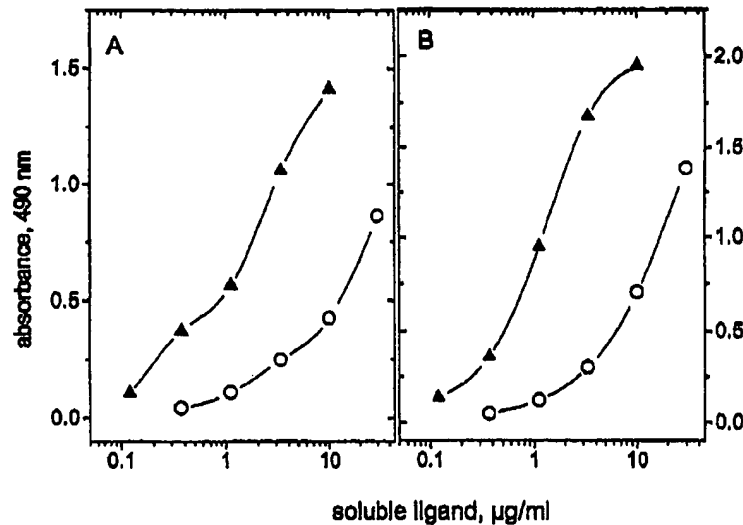


Fig. 2. Comparative binding of collagen IV and laminin-1 to immobilized $\alpha 1\beta 1$ (A) and $\alpha 2\beta 1$ (B) integrins. The soluble ligands were collagenase-solubilized collagen IV (▲) and laminin-1-nidogen complex (○) from the EHS tumor.

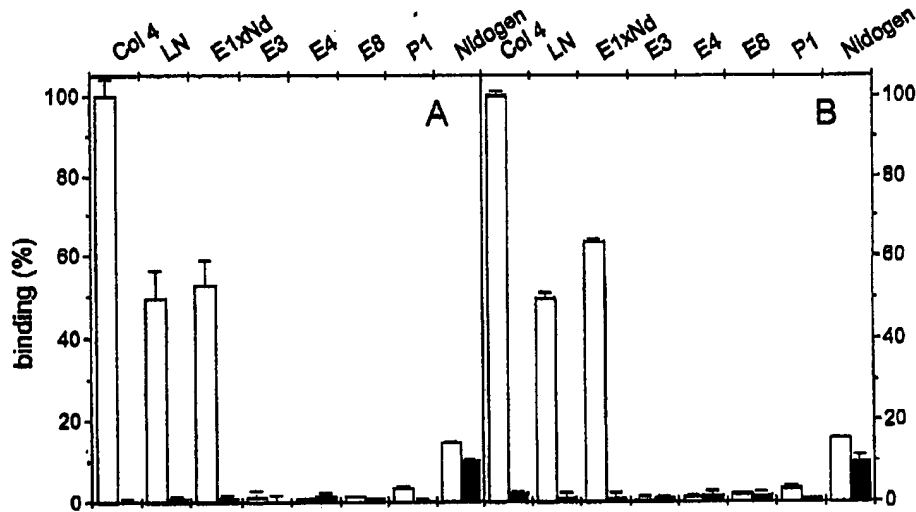


Fig. 3. Binding of fragments of EHS tumor laminin-1 and its fragments to immobilized $\alpha 1\beta 1$ (A) and $\alpha 2\beta 1$ (B) integrins. Soluble ligands (denoted at the top) were used in the absence (white bars) or presence (black bars) of 20 mM EDTA. The data are expressed relative to the binding of 10 μg/ml collagen IV (Col 4), which was set to 100% (see Fig. 2). All other soluble ligands were used at 30 μg/ml and included laminin-1-nidogen complex (LN), several laminin-1 fragments and recombinant nidogen.

of most of the short arm structures of laminin except for the distal portion of the $\beta 1$ chain (Mann et al., 1988). Fragments P1 and P1X, which lack additional distal portions, were inactive. The loss of activity was due to pepsin degradation and not to the incubation at acidic pH as shown above. In addition, no activity was usually observed for fragments E3, E4 and E8, the latter being the major cell-adhesive fragment of EHS tumor laminin-1 (Aumailley et al., 1987; Goodman et al., 1987). Some batches of the heparin-binding fragment E3 showed distinct binding to both integrins but this binding could not be inhibited by EDTA and was completely inhibited by heparin (<20 μg/ml).

The involvement of typical ligand-binding sites of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins in the interaction with fragment E1XNd was demonstrated by the sensitivity to EDTA (Fig. 3). This was further shown by specific inhibition with either anti- $\alpha 1$ or anti- $\alpha 2$ or in both cases by anti- $\beta 1$ (Fig. 4). Further inhibition assays were performed for integrin $\alpha 1\beta 1$ (15 nM) and $\alpha 2\beta 1$ (10 nM) binding to immobilized laminin-1/nidogen using soluble protein ligands. Laminin-1, fragment E1XNd and collagen IV caused, however, increased integrin-binding rather than inhibition, particularly at high concentrations (30–140 nM). This is very likely caused by laminin self assembly via its E1XNd sites (Yurchenco and Cheng, 1993).

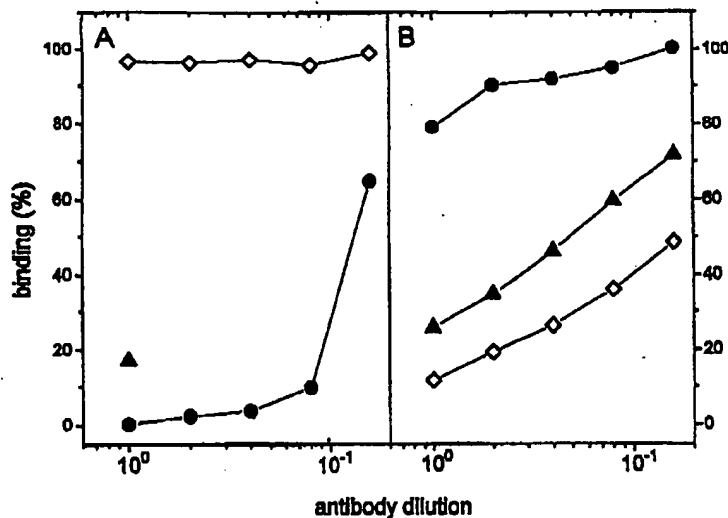


Fig. 4. Inhibition of laminin fragment E1XNd binding to immobilized $\alpha 1\beta 1$ (A) and $\alpha 2\beta 1$ (B) integrins by monoclonal antibodies against integrin subunits. The inhibitors were anti- $\beta 1$ AIB2 (▲ hybridoma supernatant, starting dilution 1:10, only this single dilution was used in A), anti- $\alpha 1$ 1B3.1 (● ascites, starting dilution 1:100) and anti- $\alpha 2$ G19 (◇ purified antibody, starting concentration 30 $\mu\text{g/ml}$).

Table 1. Activation of $\alpha 1\beta 1$ integrin and $\alpha 2\beta 1$ integrin binding to laminin fragment E1XNd with anti- $\beta 1$ (TS2/16). Binding is expressed relative to the binding in the absence of TS2/16, which was set to 100%. The corresponding absorbance values (490 nm) were 0.41 (10 $\mu\text{g/ml}$) and 0.67 (30 $\mu\text{g/ml}$) for $\alpha 1\beta 1$, and 0.05 (10 $\mu\text{g/ml}$), respectively, and 0.09 (30 $\mu\text{g/ml}$) for $\alpha 2\beta 1$. The inhibitors were added before incubation with TS2/16. Inhibiting monoclonal antibodies were those of Fig. 4 and used at 20 $\mu\text{g/ml}$ (anti- $\alpha 2$) or a 1:200 dilution (anti- $\alpha 1$).

Soluble integrin	Concentration	Inhibitor added to TS2/16	Binding
	$\mu\text{g/ml}$		%
$\alpha 1\beta 1$	10	none	180
	30	none	123
	10	10 mM EDTA	13
	10	anti- $\alpha 1$	20
	10	anti- $\alpha 2$	170
$\alpha 2\beta 1$	10	none	1450
	30	none	660
	10	10 mM EDTA	200
	10	anti- $\alpha 1$	1250
	10	anti- $\alpha 2$	120

and by collagen IV binding via nidogen (Fox et al., 1991). A smaller 70-kDa collagen IV fragment CB3, which binds both integrins (Vandenberg et al., 1991; Kern et al., 1993), inhibited laminin binding of $\alpha 1\beta 1$ (concentration causing half-maximum binding = 35 nM) and $\alpha 2\beta 1$ (concentration causing half-maximum binding = 140 nM).

As observed for laminin, the interaction with fragment E1XNd was less pronounced when the integrins were used in soluble instead of immobilized form, particularly with $\alpha 2\beta 1$. This could reflect a different status of integrin activation and we therefore used the activating anti- $\beta 1$ monoclonal antibody TS2/16 (Arroyo et al., 1992) to improve the binding activity of soluble integrins (Table 1). A dramatic increase in

binding could be observed for $\alpha 2\beta 1$ and a moderate increase for $\alpha 1\beta 1$ integrin, the increases being dependent on the integrin concentration. The activated binding of $\alpha 1\beta 1$ could be reduced to almost background levels by EDTA and anti- $\alpha 1$, indicating that both the activated and non-activated binding were due to specific integrin-ligand interactions. A similar inhibition by EDTA and anti- $\alpha 2$ was also observed for the $\alpha 2\beta 1$ interaction, but only for the contribution due to the activation step. This indicates that the low binding of non-activated $\alpha 2\beta 1$ integrin to E1XNd is non-specific. Interestingly, similar activation studies with TS2/16 antibody using immobilized $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins demonstrated no significant changes in the binding profiles for E1XNd (data not shown).

Integrin binding to a cryptic RGD site on the laminin $\alpha 1$ chain

Laminin fragment P1, which lacks affinity for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, was previously shown to become a cell-adhesive substrate (Aumailley et al., 1987) after the exposure of a cryptic RGD site by pepsin digestion (Aumailley et al., 1990b). The interaction was partially sensitive to anti- $\beta 1$ and anti- $\beta 3$ (Aumailley et al., 1991) but the α subunits involved have not yet been identified. We, therefore, examined the interaction of three RGD-dependent integrins with laminin-1 and its fragment P1. A distinct interaction was observed between immobilized $\alpha V\beta 3$ integrin and laminin fragment P1 (Fig. 5A), although it was weaker than the interaction of the integrin with vitronectin and fibronectin. No reaction was observed with EHS tumor laminin-1. Integrin $\alpha IIb\beta 3$ also bound to fragment P1 but, in addition, to a lower extent to laminin-1 (Fig. 5C). Neither laminin nor fragment P1 bound to $\alpha 5\beta 1$ integrin (Fig. 5B). The $\alpha V\beta 3$ /P1 interaction was shown to be specific since its inhibition with GRGDSP peptide was similar to that observed for other RGD-containing ligands (Fig. 6).

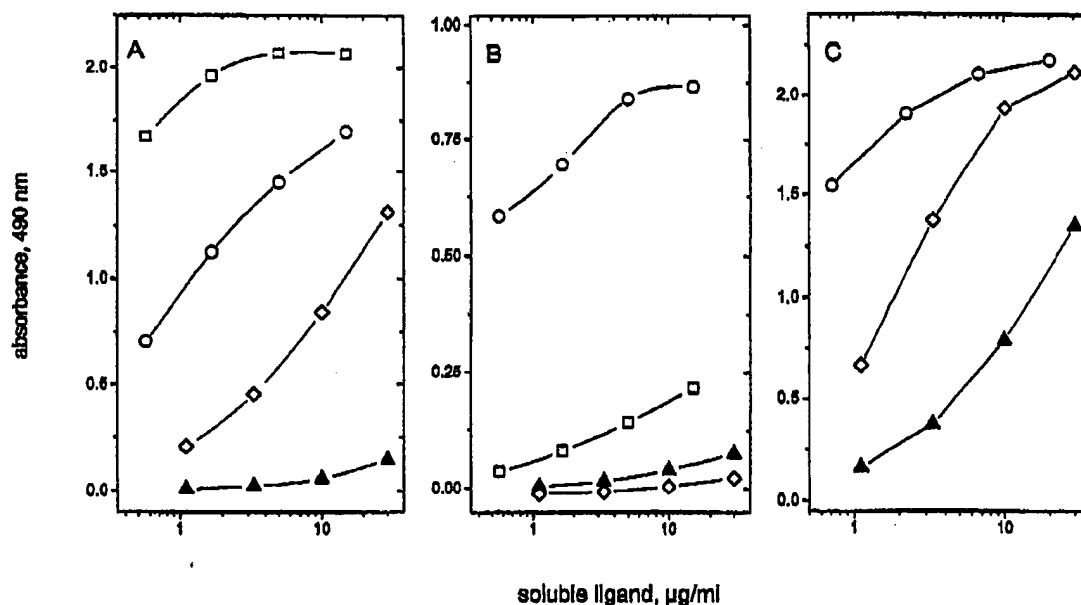


Fig. 5. Binding of various RGD-containing ligands to immobilized $\alpha V\beta 3$ (A), $\alpha 5\beta 1$ (B) and $\alpha IIb\beta 3$ (C) integrins. The ligands used were vitronectin (□), fibronectin (○), laminin fragment P1 (◇) and laminin-1-nidogen complex from the EHS tumor (▲).

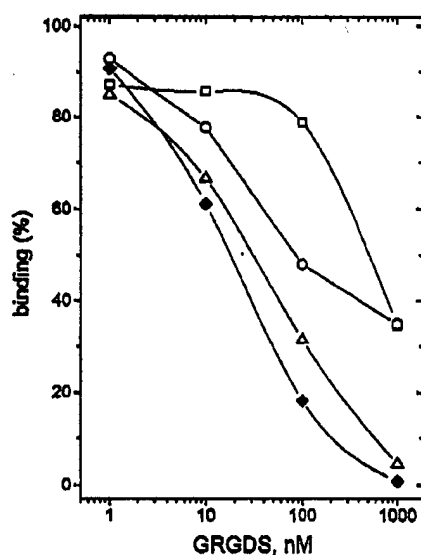


Fig. 6. Inhibition of binding of RGD-containing ligands to immobilized $\alpha V\beta 3$ integrin by synthetic GRGDS. The soluble ligands were vitronectin (□), fibronectin (○), fibrinogen (Δ) and laminin fragment P1 (◆). They were used at 10 $\mu\text{g/ml}$, except for P1 (30 $\mu\text{g/ml}$), and mixed with GRGDS before being added to $\alpha V\beta 3$. The level of binding in the absence of GRGDS was set to 100%.

Kinetic analysis of integrin binding to laminin

Biosensor technology was used for a more precise quantitative analysis of several of the integrin interactions. Laminin-1 or several of its fragments were covalently coupled via lysine residues to a dextran layer and incubated with different concentrations of integrins in the same binding buffer used

also for the solid-phase assays (see above). This revealed a distinct binding of $\alpha 2\beta 1$ integrin to both laminin-1 and fragment E1XNd with comparable rate constants and a K_D of about 50 nM (Table 2). Similar studies with immobilized collagen IV and $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins gave a K_D in the range 2–7 nM (W. Göhring and J. Eble, unpublished observations) in agreement with a similar difference observed in solid-phase binding assays (Fig. 2). Further studies demonstrated that addition of TS2/16 antibody to $\alpha 2\beta 1$ integrin caused a threefold decrease in k_{on} , which was independent of the ligand concentration used in the preceding binding step. Assuming a 1:1 stoichiometry of the soluble $\alpha 2\beta 1$ /antibody complex, a twofold higher k_{on} and a K_D of 9 nM could be calculated (Table 2). Any other stoichiometry would yield an even higher affinity either by an increase in the molecular mass (e.g. at a 2:1 ratio) or a decrease in the concentration (ratio < 1). Therefore, since this is a maximum K_D value, it indicates a clear increase in integrin affinity due to the antibody.

Kinetic and thermodynamic parameters were also obtained for $\alpha V\beta 3$ integrin binding to laminin fragment P1 and demonstrated a K_D of approximately 450 nM in two separate experiments (Table 2). However, insufficient binding of $\alpha 1\beta 1$ integrin to laminin-1 or fragment E1XNd was observed to allow a precise analysis. Since binding of $\alpha 1\beta 1$ was observed for collagen IV, this indicates that the coupling reaction has interfered with the activity of laminin.

DISCUSSION

Tomaselli et al. (1988) were the first to show that a particular cellular receptor, not identified at that time, can function as a dual collagen/laminin receptor. Since then many more reports (see Introduction) have confirmed this observation and demonstrated the involvement of two integrins, $\alpha 1\beta 1$ and $\alpha 2\beta 1$. Because of the large structural differences between

Table 2. Kinetic parameters and dissociation equilibrium constants (K_D) of integrin-laminin interactions measured by surface plasmon resonance. Ab indicates a complex with activating antibody TS2/16. For $\alpha V\beta 3$, values of two independent measurements are shown.

Soluble ligand	Immobilized ligand	k_{on}	k_{off}	K_D
		$M^{-1} s^{-1}$	s^{-1}	nM
$\alpha 2\beta 1$ integrin	laminin-1	4.48×10^4	2.6×10^{-3}	58
$\alpha 2\beta 1$ integrin	fragment E1XNd	5.86×10^4	2.9×10^{-3}	48
$\alpha 2\beta 1$ integrin/Ab	laminin-1	10.69×10^4	0.9×10^{-3}	9
$\alpha V\beta 3$ integrin	fragment P1	1.64×10^4	7.3×10^{-3}	444
		1.35×10^4	6.1×10^{-3}	452

the two protein ligands, several intriguing questions have arisen relating to the affinity and specificity of these interactions. Further data demonstrated different laminin-binding activities for $\alpha 2\beta 1$ integrin, depending on its cellular origin (Elices and Hemler, 1989; Languino et al., 1989; Kirchhofer et al., 1990) and were considered to reflect different states of integrin activation (Chen and Hemler, 1993; Kawaguchi and Hemler, 1993). In the present study, we have therefore purified $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins on a preparative scale by collagen affinity chromatography in order to study their affinity for various laminin isoforms and laminin fragments in ligand-binding assays.

Both integrins, when used in immobilized form, showed distinct binding to laminin-1 (chain composition $\alpha 1\beta 1\gamma 1$) but with a 10-fold lower strength than to collagen IV. These interactions were sensitive to EDTA and could be inhibited by antibodies to $\beta 1$, $\alpha 1$ or $\alpha 2$ integrin subunits, as can cell adhesion and ligand binding to various collagens mediated by the same integrins (Kern et al., 1993; Pfaff et al., 1993). Two human placenta laminin isoforms, laminin-2 ($\alpha 2\beta 1\gamma 1$) and laminin-4 ($\alpha 2\beta 2\gamma 1$), which differ from laminin-1 in one or two chains, showed, however, negligible binding to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. These isoforms bind to $\alpha 6\beta 1$ and more weakly to $\alpha 3\beta 1$ integrin, as has been shown with transfected K562 cells (Delwel et al., 1993; 1994), demonstrating that integrins distinguish between different laminins. Integrin $\alpha 2\beta 1$ from endothelial cells but not from platelets was reported to bind to large pepsin fragments of human placental laminins (Languino et al., 1989; Kirchhofer et al., 1990) but these fragments may originate from four different isoforms including laminin-1 (Engvall et al., 1990). The binding of endothelial $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins to laminin-1 was also shown by affinity chromatography (Kramer et al., 1990).

Another aim of our study was the mapping of ligand sites. Equal binding activities for laminin-1 and its short arm fragment E1XNd were found with $\alpha 1\beta 1$ integrin and $\alpha 2\beta 1$ integrin. This binding was specific since several other short arm (P1, P1X, E4) and long arm (E3, E8) fragments of laminin-1 and the laminin-binding nidogen were inactive ligands. The binding site for $\alpha 2\beta 1$ integrin has not been mapped before, while a few previous studies with $\alpha 1\beta 1$ integrin are in agreement with our observations. These studies were performed with the E1 fragment, which is related to E1XNd, and showed partial inhibition of choriocarcinoma cell adhesion to E1 by anti- $\alpha 1$ (Hall et al., 1990) and the binding of hepatocyte $\alpha 1\beta 1$ integrin to fragment E1 but also E8 in affinity chromatography (Forsberg et al., 1990). The latter observation for E8 is, however, at variance with our ligand-binding data. The short arms of laminin-1 are contributed by the N-terminal regions of all three chains (Engel, 1993) and appear well preserved in the elastase fragment E1XNd except for

the loss of ~600 N-terminal residues (fragments E4, E10) in the $\beta 1$ chain (Mann et al., 1988). Pepsin fragments P1X and P1, which also lack substantial distal portions (domains VI and V) of the $\alpha 1$ and $\gamma 1$ chain (Gerl et al., 1991), were inactive ligands. Furthermore, the active laminin-1 and the inactive laminin-2 differ only in the $\alpha 1$ and $\alpha 2$ chains. Together, these data suggest a localization of the binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins to the N-terminal region of the laminin $\alpha 1$ chain. Confirmation of this prediction will require binding studies with a corresponding fragment, which has so far not been generated by proteolysis but may be obtained by recombinant production.

Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind to various collagen isoforms (types I, IV and VI), which differ in chain compositions and their binding affinities. This binding requires a triple-helical configuration and unfolding of the triple helix inactivates these binding sites but exposes new sites recognized by RGD-dependent integrins (Vandenberg et al., 1991; Gullberg et al., 1992; Kern et al., 1993; Pfaff et al., 1993). The major binding sites have been localized to two different regions of collagen IV fragment CB3 (Kern et al., 1993). The same fragment also inhibits laminin binding, albeit at high concentrations, but this does not distinguish whether inhibition occurs by competition for identical binding sites or by steric hindrance. Further evidence has been provided that $\alpha 1\beta 1$ integrin requires two Asp and one Arg residue from three different collagen IV chains in precise triple-helical juxtaposition (Eble et al., 1993). From these data, it appears unlikely that the same binding structure will exist on fragment E1XNd, which implies that the two integrins have different binding sites for collagens and laminins. Whether $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins bind to the same or different sites on fragment E1XNd remains an open question. Circumstantial evidence with the BIAcore biosensor system would favour the second interpretation.

Evidence for the binding of RGD-dependent integrins to laminins has so far been scarce (Kramer et al., 1990; Aumailley et al., 1990b, 1991) and we therefore used three more purified integrins ($\alpha 5\beta 1$, $\alpha V\beta 3$ and $\alpha IIb\beta 3$) in similar binding studies. Previous studies have shown that an RGD site in the mouse $\alpha 1$ chain is cryptic in native laminin and not conserved in the human $\alpha 1$ chain, while another RGD in the latter chain has only low binding activity (Aumailley et al., 1990b). The cryptic nature of the mouse laminin $\alpha 1$ RGD was confirmed in binding studies with $\alpha V\beta 3$ integrin, which binds, although with low affinity, to the cell-adhesive fragment P1 but not to mouse laminin-1. The fibronectin receptor $\alpha 5\beta 1$ did not, however, bind to either ligand. The platelet fibrinogen receptor $\alpha IIb\beta 3$ bound not only to fragment P1 but also to some extent to laminin-1 as well as to laminin-2 and laminin-4. The $\alpha 2$ chain of the latter two laminins does

not contain an RGD sequence (Vuolteenaho et al., 1994), but $\alpha\text{IIb}\beta 3$ integrin is a receptor of broad specificity and also binds to related sequences in fibrinogen and some disintegrins (Phillips et al., 1991; Scarborough et al., 1991). A screening procedure with purified RGD-dependent integrins seems, therefore, to be useful for detecting binding sites on various laminin isoforms which may not easily be discerned in cell-adhesion assays. Whether laminin binding plays a role in platelet aggregation remains an open issue. Platelet adhesion to laminin-1 is entirely mediated by $\alpha 6\beta 1$ and not by $\alpha\text{IIb}\beta 3$ integrins (Sonnenberg et al., 1988), in agreement with our data.

The ligand-binding activity of integrins can be modulated by the cellular environment and may also differ when integrins are studied in detergent-solubilized form (Hynes, 1992; Frelinger et al., 1991). The molecular basis of these observations is not well understood. Yet it was shown that certain monoclonal antibodies against $\beta 1$ (Arroyo et al., 1992; 1993) and $\beta 3$ (O'Toole et al., 1990) integrin subunits increase affinity, probably by inducing a conformational change in the integrin. This could be demonstrated with antibody TS2/16, which increases binding of cellular or solubilized $\alpha 2\beta 1$ integrin to collagen and laminin (Arroyo et al., 1992; Chan and Hemler, 1993). This is similar to our observation that TS2/16 strongly increases the very low binding of soluble $\alpha 2\beta 1$ integrin to immobilized laminin and fragment B1XNd. A similar, although less marked, activation was observed in the binding assay with soluble $\alpha 1\beta 1$ integrin. However, no activation could be achieved in assays with immobilized integrins, even though the antibody was still able to bind to the epitope as shown by ELISA. Probably, adsorption of integrins to a plastic surface causes a comparable conformational activation which cannot be increased further by the antibody. This may also explain why solid-phase assays with immobilized integrins, as shown here, are often superior to assays with soluble integrins.

A sophisticated molecular analysis of integrin-ligand interactions will depend on the knowledge of kinetic and thermodynamic parameters. We have, therefore, used a novel biosensor technology (Fägerstam et al., 1992) for such analyses. Similar K_D values of about 50 nM were determined for $\alpha 2\beta 1$ integrin binding to immobilized laminin-1 and fragment B1XNd, in agreement with a comparable binding observed in immobilized receptor assays. The affinity was increased at least fivefold in the presence of antibody TS2/16 and most of the increase was due to a reduced dissociation rate. A similar TS2/16-mediated increase in affinity was recently reported for the $\alpha 5\beta 1$ integrin binding of U937 cells to fibronectin (Arroyo et al., 1993). The binding of $\alpha \text{V}\beta 3$ integrin to fragment P1 was, however, of low affinity ($K_D = 450$ nM) in contrast with 100-fold higher affinities determined for P1 binding to various cells (Aumailley et al., 1987). This indicates either a low level of activation for solubilized $\alpha \text{V}\beta 3$ or that other ($\beta 1$) integrins participate in cellular binding. Despite its good activity in plastic-immobilized ligand assays, soluble $\alpha 1\beta 1$ integrin did not bind to immobilized laminin in the biosensor system. This suggests that the covalent coupling of laminin to the sensor chip has inactivated the binding site for $\alpha 1\beta 1$ but not $\alpha 2\beta 1$ integrin, which in turn suggests that the binding sites for these two integrins may be different. Such inactivation is not uncommon in these assays and may be overcome by varying the coupling reaction in order to immobilize the protein ligands via different amino acid side chains (Khilko et al., 1993). This could

therefore make such biosensor assays a powerful tool in future integrin studies.

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Interaction of type IV collagen with the isolated integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$

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The triple-helical cyanogen-bromide-derived fragment CB3[IV] of collagen IV, located 100 nm from the N-terminus of the molecule, contains the binding sites for the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. To investigate the interaction of these integrins and collagen IV, we performed solid-phase and inhibition assays using as receptor isolated $\alpha 1\beta 1$ and $\alpha 2\beta 1$. The ligands used were the binding-site-bearing trimeric peptide CB3[IV] and its shorter tryptic fragments F1–F4. Using titration curves, in which the binding of soluble receptors to coated ligands and the binding of soluble ligands to coated receptors were analyzed, the binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were in different but adjacent areas of CB3[IV]. Triple-helical conformation and distinct primary structures were required for the interaction. Dissociation constants (K_d), for the affinity of integrins for collagen IV, were determined in the 1-nM range in the presence of Mn^{2+} and Mg^{2+} . In the absence of Mn^{2+} , the K_d values indicated a 30–60-fold decrease in the affinities, which for $\alpha 2\beta 1$ was further reduced by adding Ca^{2+} . In the presence of Ca^{2+} and Mg^{2+} the affinity of collagen IV for $\alpha 1\beta 1$ was four-times higher than for $\alpha 2\beta 1$.

Collagen IV is the main structural component of basement membranes (Timpl, 1989). Recently it has been shown that collagen IV exists in several different isoforms. The molecule of the major and ubiquitous form is a heterotrimer with the chain composition $[\alpha 1(IV)]_2\alpha 2(IV)$ (Dieringer et al., 1985; Vandenberg et al., 1991). Three additional minor α subunits, $\alpha 3(IV)$, $\alpha 4(IV)$ and $\alpha 5(IV)$, have been discovered, mainly in glomeruli, but also in some other basement-membrane tissues (Saus et al., 1988; Gunwar et al., 1990; Hostikka et al., 1990). $\alpha 3(IV)$ and $\alpha 4(IV)$ appear to build a heterotrimeric molecule similar to that of the major isoform (Johansson et al., 1992); the molecular organization of $\alpha 5(IV)$ is not known. The ubiquitous collagen IV, the classical form, builds a network which determines the biochemical stability and the macromolecular organization of basement membranes. In addition, it is used as a scaffold into which the other constituents of this tissue are incorporated (Yurchenco and Schittny, 1990). Another important function of collagen IV is its participation in the interaction of basement membranes with cells (Aumailley and Timpl, 1986; Vandenberg et al., 1991). The cell-surface receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ specifically mediate adhesion of cells to collagen IV (Vandenberg et al., 1991). They belong to the $\beta 1$ or very-late-antigen (VLA) integrins, a subclass of the integrin family which is mainly responsible for the interaction of cells with constituents of the extracellular matrix.

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Abbreviations. CB9[III], trimeric cyanogen-bromide-derived peptide of collagen III; CB3[IV], trimeric cyanogen-bromide-derived peptide of collagen IV; F1–F4, trypsin-derived fragments of CB3[IV]; 7S, N-terminal crosslink domain of human collagen-IV molecule; NC1, C-terminal non-collagenous globular domain of collagen-IV molecule; VLA, very-late antigen synonymous with $\beta 1$ -containing integrins.

Recently, we have localized the binding sites of the collagen-IV molecule for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ to a 150-amino-acid segment of the triple helical domain, approximately 100 nm from the N-terminal end of the molecule (Vandenberg et al., 1991). Since this segment possesses intermolecular disulfide bridges which connect the three α chains, it could be isolated in an intact triple-helical conformation after cyanogen-bromide cleavage; it is designated CB3[IV]. Further limited treatment of the CB3[IV] with trypsin, produced the shorter triple-helical segments F1–F4 (Fig. 1A), which can now be used for a more detailed investigation of the interaction between collagen IV and the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. Here we report the interaction of CB3[IV] and its trypsin-derived fragments with integrins using solid-phase and inhibition assays. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ use two distinct binding sites located on two neighbouring triple-helical segments of collagen IV. Dependent on divalent cations, affinity of collagen IV for $\alpha 1\beta 1$ was found to be higher than for $\alpha 2\beta 1$.

MATERIALS AND METHODS

Purification of receptors

The purification of $\alpha 1\beta 1$ and $\alpha 2\beta 1$, described earlier (Vandenberg et al., 1991), was slightly modified. To isolate $\alpha 1\beta 1$, homogenized and washed human placenta was extracted with buffer A (50 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 1% reduced Triton X-100 (Aldrich), 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin and antipain (1 μ g/ml each). The homogenate was centrifuged at 23000 g for 30 min and after adding 1 M $MnCl_2$ to a final concentration of 1 mM, the supernatant was applied to a CB3[IV]–Sephacrose column. The column was washed with 50 mM Tris/HCl, pH 7.4, 300 mM NaCl, 1 mM $MnCl_2$, containing 0.1% reduced Triton X-100. Triton X-100 was exchanged against *n*-octylglucoside by washing the

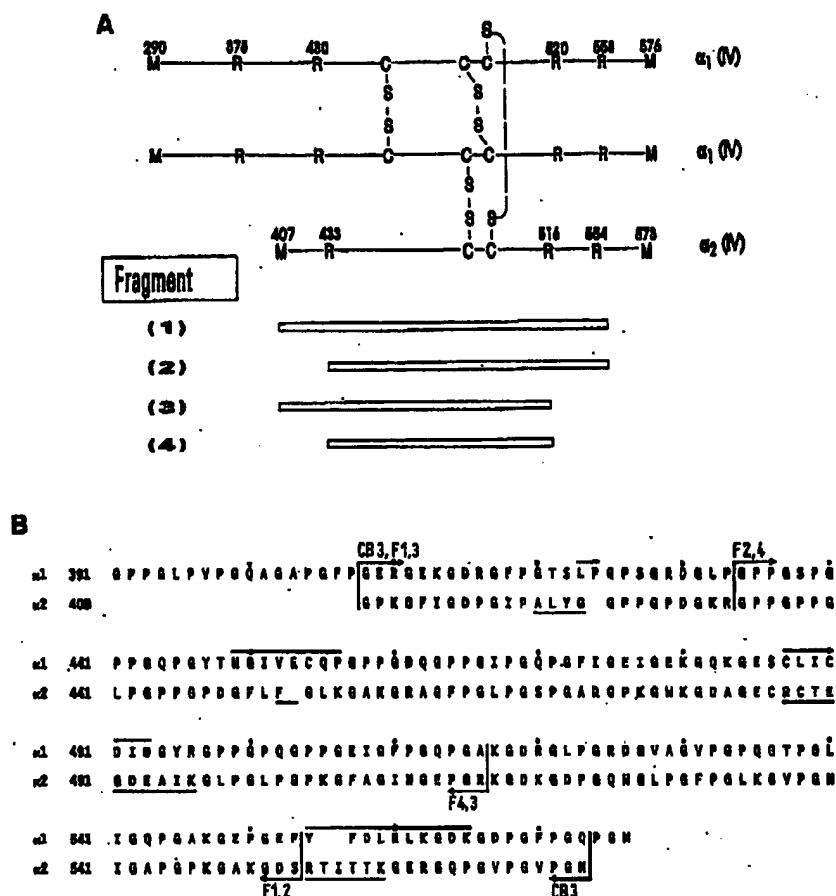


Fig. 1. Schematic representation of CB3[IV] and its tryptic fragments (according to Vandenberg et al., 1991). (A) Cyanogen bromide cleaved the $\alpha 1$ (IV) chain at Met290 and Met575 and $\alpha 2$ (IV) at Met407 and Met573. It resulted in a trimeric peptide in which the α chains are connected by disulfide bridges. Treatment of CB3[IV] with trypsin gave four short trimeric fragments. The bars indicate the length and position of the triple-helical region of F1–F4. The protruding $\alpha 1$ or $\alpha 2$ chains, at both ends of the triple helices, are not taken into account. F1, $\alpha 1$ (IV) positions 376–558 and $\alpha 2$ (IV) positions 406–554; F2, $\alpha 1$ (IV) positions 429–558 and $\alpha 2$ (IV) positions 433–554; F3, $\alpha 1$ (IV) positions 376–520 and $\alpha 2$ (IV) positions 406–516; F4, $\alpha 1$ (IV) positions 430–520 and $\alpha 2$ (IV) positions 433–516. (B) Amino acid sequence of human $\alpha 1$ (IV) and $\alpha 2$ (IV) chains covering the triple-helical region of CB3[IV] (taken from Brazel et al., 1988). The non-triple-helical interruption of $\alpha 1$ (IV) and $\alpha 2$ (IV) are indicated by lines below and above the sequences, respectively. N-terminal and C-terminal ends of the triple-helical regions of the individual fragments are designated. Position numbers of the aligned $\alpha 1$ (IV) and $\alpha 2$ (IV) chains are indicated. They do not coincide with the residue numbers of the single chains (Brazel et al., 1988). The dots above the amino acid sequence indicate every 10th position along the sequence.

column with buffer A containing 25 mM *n*-octylglucoside, 1 mM MnCl_2 and 0.02% sodium azide. Bound $\alpha 1\beta 1$ was finally eluted with buffer A containing 25 mM *n*-octylglucoside in the presence of 10 mM EDTA.

For the isolation of $\alpha 2\beta 1$, platelets were extracted as described previously (Vandenberg et al., 1991). Affinity chromatography was performed using a collagen-I–Sephacrose column (10 cm \times 2 cm, 1 mg protein/ml Sepharose) equilibrated in buffer A containing 25 mM *n*-octylglucoside, 1 mM MgCl_2 , 1 mM MnCl_2 and 0.02% sodium azide. Bound $\alpha 2\beta 1$ was eluted in the presence of 25 mM *n*-octylglucoside as described above. EDTA eluates were adjusted to 20 mM MgCl_2 , concentrated in Centricon-100 columns and washed twice with buffer A containing 25 mM *n*-octylglucoside, 1 mM MgCl_2 and 0.02% sodium azide. Protein concentration was determined using bicinchoninic acid (Pierce). As described in Vandenberg et al. (1991), purity of all receptor

preparations was analyzed by SDS/PAGE and Coomassie-blue staining. For overloaded gels, no contamination of $\alpha 1\beta 1$, with $\alpha 2$, or $\alpha 2\beta 1$, with $\alpha 1$, could be observed, nor were other proteins detected under these conditions. Since placenta extract contained, beside $\alpha 1\beta 1$, small amounts of $\alpha 2\beta 1$, we performed a Western-blot analysis of the $\alpha 1\beta 1$ preparation using a polyclonal antibody against $\alpha 2$ (kindly provided by Dr. K. von der Mark). Contamination with $\alpha 2$ could not be observed.

Purification of ligand proteins

Human collagen I and collagen IV were isolated after pepsin treatment of placenta as described (Fujii and Kühn, 1975; Timpl et al., 1981). The N-terminal cross-link domain of human collagen IV (7S) was prepared according to Kühn et al. (1981). The C-terminal non-collagenous globular do-

main (NC1) was isolated from human placenta using a two-step collagenase treatment described previously (Oberblumer et al., 1985). Human collagen III was purchased from Sigma. Pepsin-derived collagen VI (with intact triple-helical domain) murine laminin, human vitronectin and fibronectin were generously supplied by Drs. Aumailley and Timpl. Isolation of the trimeric cyanogen-bromide-derived fragment CB3[IV] and its tryptic fragments F1–F4 was performed according to Vandenberg et al., (1991) with modifications. After the first separation of the tryptic digest of CB3[IV], using a TSK 3000 SW Ultrapac column, F1 was chromatographed again using a Mono Q HR 5/5 column (Pharmacia) using a gradient of 0–2 M NaCl in a 20 mM Tris/HCl, pH 8.0 containing 2 M urea. F2–F4 were separated on a Mono S HR/5 column (Pharmacia) in 20 mM sodium acetate, pH 3.7, containing 2 M urea, applying a gradient of 0–2 M NaCl. The fragments were eluted in the order F4, F3 and F2 and their purity was checked by SDS/PAGE using 8–18% polyacrylamide gel. Reduced and non-reduced samples were included in the analysis.

The C-terminal trimeric cyanogen-bromide-derived peptide of collagen III, CB9[III], was chromatographed with CB3[IV]. The two fragments were separated on a Mono Q HR 5/5 column (Pharmacia) in 20 mM Tris/HCl, pH 8.0, containing 2 M urea with a gradient of 0–2 M NaCl.

For a complete and irreversible denaturation, collagen IV and the fragment CB3[IV] had to be denatured, reduced and carboxymethylated. The procedures described in Vandenberg et al. (1991) were used.

Biotinylation of proteins

Collagen I, CB3[IV] and F1–F4 were lyophilized and dissolved in 0.1 M sodium acetate, pH 8.3, 0.5 M NaCl. After determination of the protein concentration, a 10-fold molar excess of sulfo-succinimidyl 6-(biotinamido) hexanoate (Pierce), freshly dissolved in 0.01 M acetic acid was added. After 30 min, the reaction was terminated by adding a 20-fold excess of glycine (stock solution 1 M glycine in 0.1 M acetate, pH 8.3, 0.5 M NaCl). Reaction products of low-molecular mass were removed by ultrafiltration (Ultrafree MC, 1000 NHWL, Millipore).

Thermal denaturation

Unfolding of the triple helix, during thermal denaturation of F1–F4 and CB9[III], was recorded by CD spectroscopy. Mean molar ellipticities (θ) were monitored at 222 nm with an autodichrograph Mark IV (ISA Jobin Yvon) in a thermostated cell (Hellma) with 1-mm path length. Temperature was increased 20–60°C with a linear rate of 20°C/h using a thermostat (RKS20, Lauda) with an automatic programmer (PM351, Lauda). The fragments (approximately 100 µg/ml) were dissolved in 50 mM sodium phosphate, pH 7.4, containing 0.1 M NaCl. The degree of conversion was calculated as described by Bächinger et al. (1980).

Solid-phase assays

Collagens I, III, IV, CB3[IV], CB9[III], 7S[IV], NC1[IV] and F1–F4 were dissolved in 0.5 M acetic acid. Laminin, fibronectin, vitronectin and collagen VI were dissolved in buffer A containing 0.02% sodium azide and the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were dissolved in buffer A with 1 mM MgCl₂, 1 mM MnCl₂ and 0.02% sodium azide. For coating of the

96-well ELISA plates (Greiner), 100 µl protein solutions (2.5 µg/ml or 25 µg/ml) were added to each well and incubated for 16 h at 4°C. Only human collagen IV was coated for 1 h at room temperature. To block the remaining non-specific protein-binding sites, the microtiter plates were treated with 100 µl of buffer B (buffer A containing 1% bovine serum albumin, 25 mM *n*-octylglucoside, 1 mM MgCl₂, 1 mM MnCl₂ and 0.02% sodium azide) for 1 h and were finally washed three times with the same solution.

To analyze receptor binding, $\alpha 1\beta 1$ or $\alpha 2\beta 1$ was dissolved in buffer B, and 100 µl/well of this solution were incubated with the fixed ligand for 60 min. Non-bound receptors were removed by aspiration and washing three times with 100 µl/well buffer B. Bound receptors were first incubated with a rabbit anti-(human $\beta 1$) serum (diluted 1:200 in buffer B) for 90 min. After washing three times with buffer A containing 0.01% Tween 20, 1 mM MgCl₂, and 1 mM MnCl₂ (buffer C), the receptors were treated with goat anti-(rabbit IgG) coupled to horseradish peroxidase (Sigma), diluted 1:800 in buffer C for 90 min. Finally, peroxidase activity was measured using a staining kit from Pierce with tetramethylbenzidine as substrate. Non-specific binding, which varied between 3% and 4%, was measured in the presence of 10 mM EDTA. Absorption was measured at 450 nm using an ELISA Reader (MR 600, Dynatech). All experiments were performed in triplicate.

In an inhibition assay, binding of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ to collagen IV was prevented by CB3[IV] and F1–F4. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were dissolved in buffer B and incubated for 30 min with increasing concentrations of CB3[IV], F1, F2, F3 or F4 in buffer A. The concentration of the receptor protein was 2.5 µg/ml. 100 µl each mixture was then added to wells coated with collagen IV. After 60 min, non-bound proteins were removed by washing the wells with buffer B and bound receptors were detected as described above. Each experiment was performed in duplicate.

For binding experiments with biotinylated fragments of collagen IV (modified according to Charo et al., 1990), $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were coated onto plastic (coating concentration 2.5 µg/ml, 100 µl/well). After blocking non-specific-binding sites with buffer A containing 1% bovine serum albumin, 1 mM MgCl₂, 1 mM MnCl₂ and 0.02% sodium azide (buffer D), serial dilutions of CB3[IV], F1, F2, F3 or F4 in buffer D were incubated with the fixed receptors for 120 min at room temperature. After washing the wells three times with buffer D, bound biotinylated proteins were detected with horseradish peroxidase coupled to streptavidin (Sigma, 1 µg/ml) dissolved in buffer D (100 µl/well). After 60 min, the wells were washed three times with buffer A and bound peroxidase activity was measured as mentioned above. Non-specific binding of biotinylated CB3[IV] was determined by inhibition with a 40-fold excess of non-labelled CB3[IV]. Similar results were obtained in the presence of 10 mM EDTA. Non-specific binding of F1–F4 was measured in the presence of 10 mM EDTA. It did not exceed 10%. All experiments were performed in triplicate.

To determine the number of receptor molecules immobilized on the plastic surface, the coating efficiency of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ was analyzed. The receptors were radiolabelled using Na¹²⁵I (Amersham) and Iodobeads (Pierce). Iodine which did not bind was removed by ultrafiltration. The iodinated integrins were added to non-radiolabelled $\alpha 1\beta 1$ and $\alpha 2\beta 1$, dissolved in buffer A containing MnCl₂ and MgCl₂ (final receptor concentration 2.5 µg/ml). After coating, the bound receptors were removed from microtiter plates with

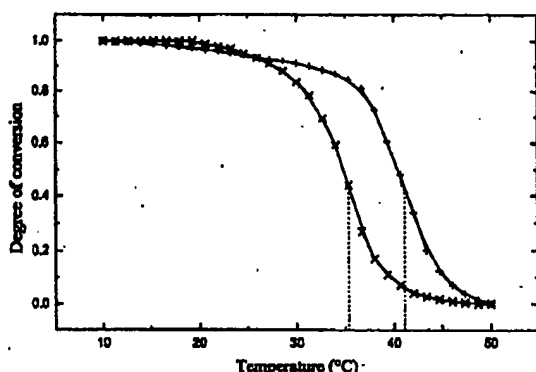


Fig. 2. Thermal-denaturation profiles of bovine collagen III (+) and human CB9[III] (x). Preparations were soluble in 0.05 M sodium phosphate, pH 7.4, containing 0.1 M NaCl. Profiles were recorded using circular dichroism at 222 nm and a linear temperature gradient of 20°C/h. The melting temperatures (t_m) are indicated (---). Degree of conversion $\Delta\theta/\Delta\theta_{max}$.

0.5 M NaOH and 0.1% SDS. Radioactivity was measured in a scintillation counter (Cobra Auto-gamma, Packard). The coating efficiency of both receptors was determined to be 50–60%.

The coating efficiency of collagen IV and CB3[IV] was compared as described in Vandenberg et al. (1991). The values were comparable and varied 30–50%.

Antibodies against the human integrin- $\beta 1$ subunit

$\alpha 2\beta 1$ was separated on SDS/PAGE (7.5% acrylamide) and protein bands were visualized by a short staining for 15 min with 0.25% Coomassie blue in 50% ethanol, 10% acetic acid. Bands corresponding to the $\beta 1$ subunit were excised from the gels, homogenized with complete Freund's adjuvant and injected into rabbits. Booster injections were made after four weeks with $\beta 1$ -containing bands and after eight weeks with complete $\alpha 2\beta 1$. Blood was collected two weeks after each injection and tested in ELISA assays.

RESULTS

$\alpha 1\beta 1$ and $\alpha 2\beta 1$ interact with triple-helical collagen IV

Adhesion of cells, such as HT1080 (human fibrosarcoma) or RuGli (rat glioblastoma) to CB3[IV] (Vandenberg et al., 1991), containing the major cell-binding site of collagen IV, depends on an intact triple helix. There are, however, reports that cells can also adhere to non-triple-helical fragments and short peptides of collagen IV (Chelberg et al., 1990; Wilke and Furcht, 1990). We investigated whether the isolated integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are able to differentiate between an intact triple-helical structure and unfolded α chains. We found that soluble $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bound to native collagen IV, but not to denatured collagen IV, coated onto a plastic surface (data not shown). Similar experiments have been performed with native and denatured CB3[IV] which contain the major recognition sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$. No binding of the two receptors to denatured CB3[IV] could be observed (data not shown).

$\alpha 1\beta 1$ and $\alpha 2\beta 1$ are the typical collagen receptors of the integrin family. They bind to collagen IV as well as to several other collagen types (Tomaselli et al., 1988; Kramer and

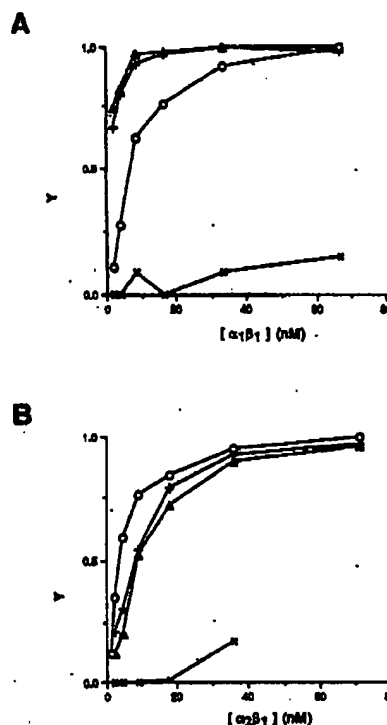


Fig. 3. Binding of (A) $\alpha 1\beta 1$ and (B) $\alpha 2\beta 1$ to collagen III (+), collagen IV (A), CB3[IV] (O) or CB9[III] (X) coated onto plastic for 90 min (25 μ g/ml, 100 μ l/well). After incubation for 90 min at increasing concentrations of $\alpha 1\beta 1$ and $\alpha 2\beta 1$, bound receptors were detected by ELISA using antibodies against $\beta 1$. Colour development was measured at 450 nm using tetramethylbenzidine as peroxidase substrate. Non-specific binding, determined in the presence of 10 mM EDTA was subtracted. Fractional occupancy Y, the ratio of actual absorbance (A) to maximal absorbance (A_{max}), is plotted versus concentration of receptor. Data represent means of triplicates, deviation below 10%.

Marks, 1989; Clyman et al., 1990; Staatz et al., 1989; Ignatius et al., 1990), e.g. the fiber forming collagens I and III (Fig. 5A and B). The question arose, as to whether the triple-helical structure is sufficient to bind $\alpha 1\beta 1$ and $\alpha 2\beta 1$ or whether additional sequence information is necessary. We therefore compared the binding capacity of the two integrins to the entire collagen III and its trimeric C-terminal cyanogen-bromide-derived peptide CB9[III]. Since the $\alpha 1(III)$ chains are disulfide bridged at the C-terminal end, CB9[III] was expected to refold to a triple-helical conformation after isolation. Before the binding experiments were performed, the triple-helical conformation of CB9[III] had to be examined by CD spectroscopy. CB9[III], as well as the entire collagen-III molecule, exhibited at 222 nm the characteristic positive molar ellipticity of an intact triple helix. CD at 222 nm was then used to measure the transition curves of the two collagen preparations. Although the denaturation temperature of the CB9[III] fragment was 3°C lower than that of the entire molecule, it clearly possesses a triple-helical conformation at 25°C (Fig. 2). The binding experiments revealed that the two receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bound to collagen III as well as to collagen IV, whereas an interaction with the fragment CB9[III] could not be observed (Fig. 3A and B).

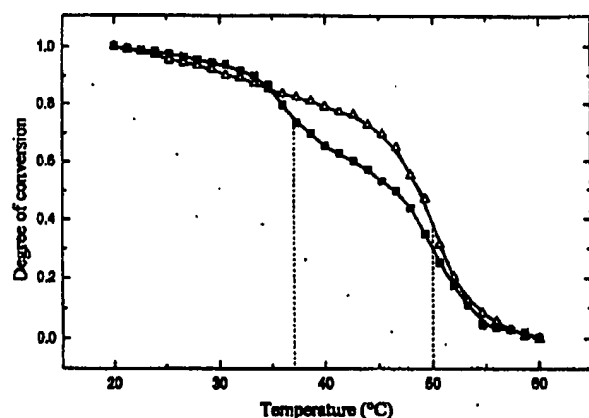


Fig. 4. Thermal-denaturation profiles of F3 (■) and F4 (△). Experimental details are described in legend to Fig. 2. The two melting temperatures, $t_m = 37^\circ\text{C}$ and $t_m = 50^\circ\text{C}$, are indicated.

In the same manner as described for collagen III and CB9[III], the stability of the triple-helical conformation of CB3[IV] and F1–F4 was investigated (Fig. 4). With the exception of F4, F1, F2 (data not shown) and F3 showed a biphasic melting curve with two transition temperatures (t_m) of 37°C and 50°C . The higher t_m value could be ascribed to F4, the central part of F1, which is stabilized by intramolecular disulfide bonds. The lower t_m value reflected the thermal stability of the two terminal triple-helical segments. All binding experiments with $\alpha 1\beta 1$, $\alpha 2\beta 1$ and the ligands were performed at 25°C , which ensured an intact triple-helical conformation of the collagen-IV fragments.

Binding of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ to a variety of extracellular matrix constituents

We compared the amounts of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ which bind to coated collagens I, III, IV and VI and to the non-collagenous adhesive proteins fibronectin, vitronectin and laminin, using an ELISA assay with antibodies against the $\beta 1$ subunit present in both integrins. $\alpha 1\beta 1$ showed the highest affinity for collagen IV (Fig. 5A), whereas $\alpha 2\beta 1$ had high affinity for collagen I (Fig. 5B). Collagen VI exhibited a relatively low affinity for $\alpha 1\beta 1$ and no affinity for $\alpha 2\beta 1$. Binding to non-collagenous proteins was not observed under the conditions used. Only laminin was an exception, with a low but distinct binding capacity for $\alpha 1\beta 1$ (Fig. 5A). As expected from cell-adhesion experiments, the terminal NC1 and 7S domains of collagen IV did not show any affinity for the two integrins.

Interaction of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ with CB3[IV] and F1–F4

To analyze the interaction of integrins with the different collagen-IV fragments, two series of experiments were performed. The first one was designed as an inhibition assay. Collagen IV was coated onto plastic and the binding of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ to the fixed collagen IV was inhibited by the different fragments. In the case of $\alpha 1\beta 1$, the reaction of collagen IV was completely inhibited by all five fragments (Fig. 6A). With $\alpha 2\beta 1$, only CB3[IV], F1 and F3 were able to inhibit F4, lacking the N-terminal and C-terminal triple-helical segment of F1, did not show any inhibitory activity. F2, which contains in addition to the C-terminal triple-helical segment of F1 only the weak recognition site for $\alpha 2\beta 1$, exhibited lim-

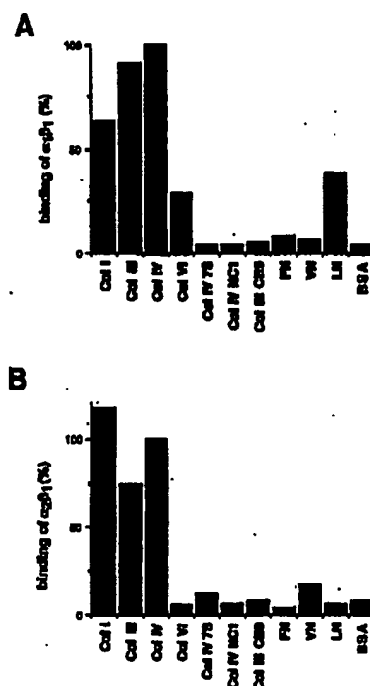


Fig. 5. Binding of (A) $\alpha 1\beta 1$ and (B) $\alpha 2\beta 1$ to coated extracellular-matrix proteins (collagens I, III, IV and VI), the fragment of collagen IV (7S, NC1), the collagen-III fragment (CB9[III]), the adhesive proteins fibronectin (FN), vitronectin (VN), laminin (LN) and bovine serum albumin (25 $\mu\text{g}/\text{ml}$, 100 $\mu\text{l}/\text{well}$). 0.5 $\mu\text{g}/\text{well}$ $\alpha 1\beta 1$ or $\alpha 2\beta 1$ were incubated for 90 min. Bound receptors were determined as described in the legend of Fig. 2. Absorbance at 450 nm of $\alpha 1\beta 1$ -bound collagen IV was set to 100%. Data represent means of triplicates.

ited inhibition capacity (Fig. 6B), less than one would expect from the binding curve shown in Fig. 8B. The reason for this discrepancy may be that the maximal concentration of 500 nM F2, used in the inhibition assay, was not high enough to reveal an optimal effect.

To complement the results of the inhibition assays using a second series of experiments, the receptors were coated on plastic and the binding of increasing amounts of biotinylated fragments was analyzed. Biotinylation did not interfere with the specificity of ligand-receptor interaction; it increased, however, to a small extent non-specific interaction with the receptor. Thus binding of biotinylated CB3[IV] to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ was almost completely inhibited by a 40-fold excess of non-labelled material (Fig. 7). The low binding activity, which was still observed, represented non-specific binding, since the same rest activity was found in the presence of 10 mM EDTA, which inactivates the receptor. Non-specific binding of biotinylated F1–F4 was then determined in the presence of EDTA. Binding of the different ligands to $\alpha 1\beta 1$ and $\alpha 2\beta 1$, as measured by titration, correlated essentially with inhibition experiments discussed above. Thus all fragments bound to the same extent to $\alpha 1\beta 1$ (Fig. 8A), whereas $\alpha 2\beta 1$ had a significantly lower affinity or no affinity for F4 and F2, respectively (Fig. 8B).

According to the method of Heyn and Weischet (1975), the saturation curves were linearized. The slope of the straight line gives $1/K_d$. The amount of binding sites is calculated from the negative intercept of the y-axis. 0.2–0.3 pmol

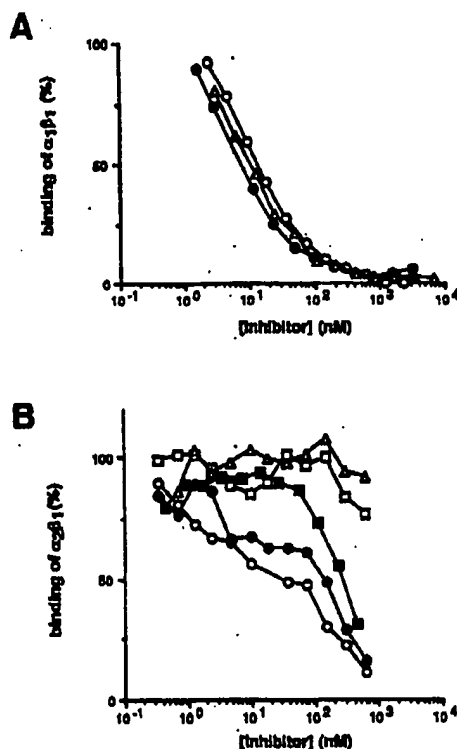


Fig. 6. Capacity of CB3[IV] and F1-F4 to inhibit the binding of (A) $\alpha 1\beta 1$ and (B) $\alpha 2\beta 1$ to collagen IV. The integrins (2.5 $\mu\text{g}/\text{ml}$) were incubated with increasing amounts of CB3[IV] (○), F1 (●), F2 (□), F3 (■) and F4 (Δ) for 30 min before adding to coated collagen (5 $\mu\text{g}/\text{ml}$, 100 $\mu\text{l}/\text{well}$). After a 60-min incubation bound receptors were detected as described in the legend of Fig. 3. Absorbance of bound receptor in the absence of inhibitor was set to 100%. Data represent means of duplicates. In Fig. 6A, inhibition curves of F2 and F3 are not shown, for reasons of clarity.

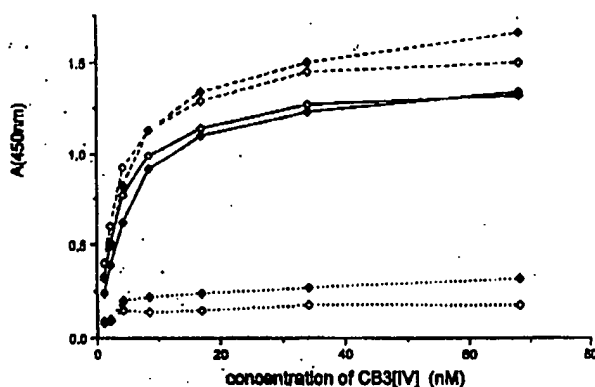


Fig. 7. Binding of biotinylated CB3[IV] to $\alpha 1\beta 1$ (○) and $\alpha 2\beta 1$ (●). The receptors were coated onto plastic (0.45 pmol immobilized receptor/well). Biotinylated CB3[IV] was incubated for 2 h in the presence (.....) or absence (—) of a 40-fold excess of non-biotinylated CB3[IV]. Bound biotinylated CB3[IV] was determined using peroxidase-coupled streptavidin and tetramethylbenzidine as substratum. Specific binding (—) was calculated as the difference of total and non-specific binding. Data represent means of triplicates.

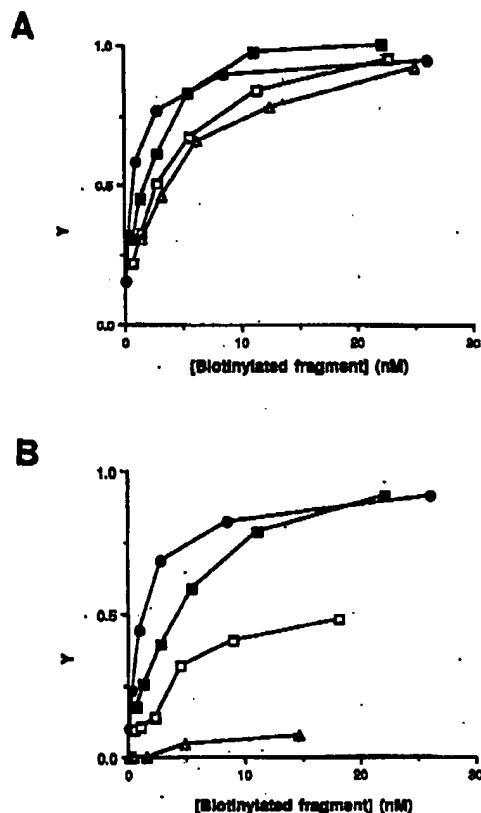


Fig. 8. Specific binding of biotinylated F1 (●), F2 (□), F3 (■) and F4 (Δ) to coated (A) $\alpha 1\beta 1$ or (B) $\alpha 2\beta 1$ (8 nmol/well) in the presence of 1 mM MnCl_2 and 1 mM MgCl_2 . Non-specific binding (less than 10%), determined in the presence of 10 mM EDTA, was subtracted. After a 2-h incubation, bound fragments were determined as described in Fig. 7. Data represent means of triplicates. Fractional occupancy (Y) was calculated as described in the legend to Fig. 3.

$\alpha 1\beta 1$ or $\alpha 2\beta 1$ /well were active in binding the collagen-IV ligands, which is approximately 50% of the protein immobilized during coating. In the presence of Mn^{2+} , which increases the affinity of several integrins for their ligands, the affinity of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ for the fragments was similar to K_d values of about 1 nM. Omission of Mn^{2+} reduced the affinity of CB3[IV] for $\alpha 2\beta 1$ to a greater extent than for $\alpha 1\beta 1$ (Table 1). Addition of Ca^{2+} , to resemble the *in vivo* situation, had no effect on the binding of CB3[IV] to $\alpha 1\beta 1$. However, it did reduce the affinity of this fragment for $\alpha 2\beta 1$. Under these conditions the affinity of collagen IV for $\alpha 1\beta 1$ is four times higher than for $\alpha 2\beta 1$.

DISCUSSION

The aim of our investigations was to analyze the interaction of the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ with the triple-helical-binding sites of collagen IV in detail. For this purpose, the investigation of cell adhesion to collagen IV proved to be limited. Although adhesion of the $\alpha 2\beta 1$ -containing HT1080 cells to CB3[IV] could be inhibited completely by antibodies against CB3[IV], attachment of these cells to the entire collagen-IV molecule was inhibited 70%, so that 30% of cell

Table 1. Dissociation constants (K_d) calculated according to Heyn and Welsch (1975), from titration curves of biotinylated CB3[IV] and F1–F4 bound to coated $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (see Fig. 7 and Fig. 8). Ligand/receptor interaction occurred in the presence of 25 mM *n*-octylglucoside, 1 mM $MnCl_2$ and 1 mM $MgCl_2$. The binding of CB3[IV] to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ was additionally measured in the absence of $MnCl_2$, in the presence of 1 mM $MgCl_2$, or in the presence of 1 mM $MgCl_2$ and 2 mM $CaCl_2$. —, not determined.

Frag- ment	K_d for integrin					
	$\alpha 1\beta 1$ with Mn^{2+} and Mg^{2+}	$\alpha 2\beta 1$ with Mn^{2+} and Mg^{2+}	$\alpha 1\beta 1$ with Mg^{2+}	$\alpha 2\beta 1$ with Mg^{2+}	$\alpha 1\beta 1$ with Mg^{2+} and Ca^{2+}	$\alpha 2\beta 1$ with Mg^{2+} and Ca^{2+}
	nM					
CB3[IV]	1	1–2	30	60	25	110
F1	1	1–3	—	—	—	—
F2	1	20	—	—	—	—
F3	1	1–2	—	—	—	—
F4	1	300	—	—	—	—

binding was caused by unknown effects (Vandenberg et al., 1991). We therefore performed these experiments with the isolated integrin receptors and used CB3[IV] and F1–F4 as ligands. With this receptor/ligand combination, we were able to show that interaction between $\alpha 1\beta 1$ or $\alpha 2\beta 1$ and collagen IV could be completely inhibited by CB3[IV], which finally demonstrated that CB3[IV] contains all the essential binding sites of collagen IV for the two integrins. However, solid-phase assays are artificial systems and do not always reflect the *in vivo* situation. Thus, it has been shown that integrins, separated from their natural environment, can alter to some extent their specificity (Cheresh et al., 1987; Conforti et al., 1990; Santoro, 1989; Stallcup et al., 1989; Elices et al., 1990).

We have performed the solid-phase assays in two different ways. Either the ligand collagen IV and its fragments or the receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were coated onto the plastic surface and the binding of the soluble counterparts was analyzed. The first system was mainly used for inhibition assays, where the inhibitor/receptor interaction occurred in solution. Using the second system, we first determined whether the binding capacity of the receptor is impaired by fixation onto plastic. Comparing the number of receptor molecules immobilized on the plastic surface with the moles of ligands bound, it was established that 50% of the receptors were still active, assuming a stoichiometry of 1:1 for the receptor/ligand complex. Using these two systems, analysis of the interaction of collagen IV, CB3[IV] and F1–F4 with $\alpha 1\beta 1$ and $\alpha 2\beta 1$ led qualitatively and quantitatively to comparable results. They were also in agreement with cell-adhesion experiments, in which binding of HT1080 cells, containing $\alpha 2\beta 1$, and RuGII cells, containing $\alpha 1\beta 1$, to collagen IV and its fragments were analyzed (Vandenberg et al., 1991).

These studies enabled us to locate the binding site of collagen IV for $\alpha 1\beta 1$ on fragment F4, representing a 74-amino-acid triple-helical segment. Due to intramolecular disulfide bridges within F4, this binding site appears to be particularly stable against unfolding and presumably also against proteolytic degradation. For $\alpha 2\beta 1$, two binding sites, a major and a minor one, were detected in the area of F1. The major one resides at the 26-amino-acid triple-helical segment of F1, while the minor one, with a 20-fold lower affinity, is located

at the C-terminal triple-helical part of F1, which is 37-residues long. Both binding sites seem to be independent from each other, since F1 contains both $\alpha 2\beta 1$ -specific sites and does not show a higher affinity than F3, which comprises only the major one.

The titration curves of soluble ligands against coated receptors or of soluble receptors against fixed ligands were used to calculate dissociation constants. In the presence of Mn^{2+} , known to stabilize the interaction between distinct integrins and extracellular-matrix ligands (Gallit and Ruoslahti, 1988; Kirchhofer et al., 1990b) and Mg^{2+} , the affinity of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ for CB3[IV] was found to be in the 1-nM range. Removal of Mn^{2+} had a greater effect on $\alpha 2\beta 1$ than on $\alpha 1\beta 1$. Without Mn^{2+} and only in the presence of Mg^{2+} , the affinity of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ for collagen IV decreased 30-fold and 60-fold, respectively. After addition of Ca^{2+} to incubations containing Mg^{2+} , a further 2-fold reduction of the affinity of $\alpha 2\beta 1$ for CB3[IV] was observed. In the presence of Mg^{2+} and Ca^{2+} , similar to the *in vivo* situation, the affinity of $\alpha 1\beta 1$ for collagen IV was five-times higher than for $\alpha 2\beta 1$. Similar inhibitory or regulatory effects of Ca^{2+} were observed for the adhesion of platelets to collagen and the binding of $\alpha 2\beta 1$ to collagen ligands (Santoro, 1986; Grzesiak et al., 1992).

$\alpha 1\beta 1$ and $\alpha 2\beta 1$ are described as typical collagen and laminin receptors (Tomaselli et al., 1988; Kramer and Marks, 1989; Clyman et al., 1990; Staats et al., 1989; Ignatius et al., 1990; Hynes, 1987; Hall et al., 1990; Languino et al., 1989). To test our system, we compared the binding of the two integrins to several extracellular constituents. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bound best to collagen IV and the fiber forming collagens. As expected, laminin reacted with $\alpha 1\beta 1$ but not with $\alpha 2\beta 1$, and this is in agreement with observations that for platelets $\alpha 2\beta 1$ is specific for collagen IV but not for laminin (Elices and Hemler, 1989; Kirchhofer et al., 1990a). For other cells $\alpha 2\beta 1$ recognized both ligands. Adhesive proteins, such as fibronectin and vitronectin, exhibited no affinity for these two integrins.

The interaction of $\alpha 2\beta 1$ with collagen I has been investigated in detail because this reaction is responsible for the adhesion of platelets, the initial step of platelet aggregation (Santoro et al., 1988; Kunicki et al., 1988; Staats et al., 1989; Takada and Hemler, 1989). A major binding site of collagen I for $\alpha 2\beta 1$ has been located within the cyanogen-bromide-derived peptide $\alpha 1(III)CB3$ (Staatz et al., 1990) and the essential amino acid sequence was determined to be K D G E A (Staatz et al., 1991). Also $\alpha 1\beta 1$, from hepatocytes and fibroblasts interacts with collagen I via $\alpha 1(III)CB3$ (Gullberg et al., 1989 and 1990). The exact binding site has not been investigated in detail. Collagen III is also involved in platelet adhesion. Its major interaction site was located within the N-terminal cyanogen-bromide-derived peptide $\alpha 1(III)CB3$, containing the sequence K D G E S, very similar to the active sequence in collagen I (Morton et al., 1991). Significantly, these or similar sequences were not found in collagen IV, neither in the 26-residue region within the major binding site of collagen IV for $\alpha 2\beta 1$, nor in the entire fragment CB3[IV] (Fig. 1B; Vandenberg et al., 1991). Recently, the fibril forming collagen from the tube worm *Riftia pachyptila* was also shown to bind cells although it does not contain the sequence D G E A (Mann et al., 1992).

From the experiments presented here, we can deduce that two facts are important for the interaction of collagen IV with integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. These are, firstly, an intact triple-helical conformation and, secondly, some definite se-

quence information. The triple helix is a necessary but not sufficient prerequisite. Although F4 has an intact stable triple helix, it does not interact with $\alpha 2\beta 1$. The binding sites of the homologous $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are independent from each other in spite of their close vicinity. The distinct primary structures of the two triple-helical segments select specifically either for $\alpha 1\beta 1$ or $\alpha 2\beta 1$. The question arises, whether this is also true for other collagens. At high molecular excess, it has been observed that cyanogen-bromide-derived peptides, with apparently no intact triple-helical structure, interact with cells or with isolated integrins (Statz et al., 1991; Gullberg et al., 1989). It is, however, usual that this kind of reaction is more effective under conditions where at least a partial refolding of the triple-helical structure can occur (Morton et al., 1991) and that no activity is observed when refolding is impossible.

To what extent the primary structure of the triple-helical-collagen ligands selects for $\alpha 1\beta 1$ or $\alpha 2\beta 1$ is not entirely clear. Significantly, the triple-helical segments comprising different amino acid sequences appear to interact with the same integrin. This indicates a relatively broad specificity of the integrins, which may be modulated from ligand to ligand. Closer inspection of the two-dimensional or three-dimensional distribution of functional amino acids on the surface of the triple helix may give us a better insight into the principles of integrin/ligand interaction. An experimental approach to solve this problem could be the analysis of cross-links of the complexes between triple helices and integrins.

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Chondrocyte and Chondrosarcoma Cell Integrins with Affinity for Collagen Type II and Their Response to Mechanical Stress

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Mechanical stress is an important regulator of chondrocyte functions but the mechanisms by which chondrocytes sense mechanical signals are unknown. Receptors for matrix molecules are likely involved in the mechanical signaling. In the first part of this study we identified integrins with affinity for the cartilage-specific collagen type II. We report that the collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ isolated from bovine chondrocytes or human chondrosarcoma cells bound collagen type II as judged from affinity chromatography. The integrins $\alpha 3\beta 1$ or $\alpha 9\beta 1$ did not bind collagen type II-Sepharose. In the second part of the study we investigated the effect of mechanical stress on expression of matrix molecules and integrin subunits. Chondrocytes and chondrosarcoma cells, cultured on uncoated flexible silicone membranes in the presence of serum, were exposed to mechanical stress by the Flexercell system. Dynamic stimulation of chondrocytes for 3 h increased the mRNA expression of collagen type II and aggrecan as judged by Northern blotting, while the $\beta 1$ -integrin subunit was not changed. When chondrosarcoma cells were exposed to mechanical stimulation under the same conditions, mRNA expression of $\alpha 5$ was found to increase while $\beta 1$, $\alpha 2$, and αv did not increase to significant levels. In another study the effect of mechanical stress on integrins was investigated when the cells were cultured on collagen type II-coated flex-dishes. Three hours of dynamic stress increased the mRNA expression of $\alpha 2$ -integrin subunit while the level of mRNA for integrin subunits $\beta 1$, $\alpha 1$, $\alpha 5$, and αv showed no or small changes, indicating that matrix components may modulate the expression of integrins during mechanical stress. © 1995 Academic Press, Inc.

INTRODUCTION

Chondrocytes are the only type of cells present in cartilage. They play a key role in cartilage homeostasis,

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controlling normal turnover of matrix molecules, integrating the molecules into a functional matrix, and responding to changes in load with appropriate remodeling. The biomechanical properties of the highly specialized cartilage extracellular matrix depend on the structure and organization of the two major constituents, collagens and proteoglycans [1, 2], where collagen type II represents around 95% the collagens [3] and aggrecan is prominent among the proteoglycans [4, 5]. Some of the cartilage proteins, such as collagen types II and VI, fibronectin, and chondroadherin (36 kDa), have been shown to mediate adhesion of isolated chondrocytes and may thus have important functions in the communication between chondrocytes and the extracellular matrix [6–8].

Mechanical load is an important regulator of chondrocyte metabolism and is a prerequisite for maintaining normal cartilage matrix properties (reviewed by Urban [9]). It is known that normal dynamic load stimulates the synthesis of collagen type II and proteoglycans while a static load decreases this synthesis [10, 11]. Mechanical load may also affect the degradation of cartilage [9]. The mechanisms by which mechanical signals are transformed into intracellular signals are not understood; however, cell surface receptors for matrix molecules are likely involved in these processes.

Integrins are of prime importance for the adhesion of many types of cells to extracellular matrix proteins [12–14]. They consist of two subunits, α and β , and the extracellular domains of the α subunits have several divalent cation-binding sites. The integrins are excellent candidates for mechanoreceptors since they interact with actin-binding proteins and thereby link the extracellular matrix with the cytoskeleton [15, 16]. Wang and co-workers have shown that $\beta 1$ -integrins supported a stress-dependent stiffening response of the cytoskeleton when mechanical stresses were applied with a magnetic twisting device [17], indicating that $\beta 1$ -integrins are indeed able to transmit mechanical signals.

The major collagen type I-binding integrins are $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [18–20] while $\alpha 3\beta 1$ and $\alpha 9\beta 1$ have been reported to interact with this ligand but apparently with

lower affinity [21, 22]. Little is, however, yet known concerning the collagen-binding integrins in cartilage. It has been reported that $\alpha 2\beta 1$ from fetal bovine chondrocytes is affinity purified on collagen type II-Sepharose [23].

Recent studies have shown that articular chondrocytes express a number of integrins [23–26] but their ligands in cartilage are not fully defined. In the present study we identified chondrocyte integrins with affinity for collagen type II and in addition we investigated changes in expression of mRNAs encoding collagen type II, aggrecan, and various integrin subunits in response to dynamic mechanical stress.

MATERIALS AND METHODS

Materials. Collagenase (type II) was obtained from Worthington. Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, lactoperoxidase, glucoseoxidase, and rabbit anti-mouse IgG-agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Protease-free testicular hyaluronidase was from Leo Pharmaceuticals, Helsingborg, Sweden. CNBr-activated Sepharose, protein A-Sepharose and mRNA Quick-prep were from Pharmacia, Uppsala, Sweden. Na^{125}I was from New England Nuclear (Boston, MA). Collagen type II was isolated from nasal cartilage by pepsin digestion [27]. Dulbecco's culture medium, MEM, MEM α , F12, EBSS, fetal calf serum (FCS), trypsin, penicillin, and streptomycin were purchased from GIBCO and culture dishes were obtained from Nunc, Denmark. Culture dishes with flexible surfaces were from Flexcell Corp. (PA).

Antibodies. The monoclonal antibody against human $\alpha 1$ -integrin (TS27) was a generous gift from T. Springer, Boston, and monoclonal antibodies against the human integrin subunits $\beta 1$ (P4C10), $\alpha 2$ (P1E6), $\alpha 3$ (P1B5), $\alpha 5$ (P1D6), and $\alpha \nu$ (VNR147) were from Life Technologies Inc. (Grand Island, NY). The monoclonal antibody against human $\alpha 2$ -subunit (G119), with cross-reactivity against bovine $\alpha 2$ -integrin, was a kind gift from Dr. Sentot Santoso, Giessen, Germany. Rabbit polyclonal antibodies against rat $\beta 1$ -integrin was a kind gift from Kristofer Rubin (Uppsala, Sweden), [28] and rabbit polyclonal antibodies against human $\alpha 1$ was from Calbiochem (La Jolla, CA).

cDNA probes. cDNAs for human $\text{pro}\alpha 1(\text{I})$ and $\text{pro}\alpha 1(\text{II})$ collagens, bovine aggrecan, and human $\alpha 1$ -integrin were kindly provided by Dr. E. Vuorio [29], Dr. Å. Oldberg [30], and Dr. E. Marcantonio [31], respectively. cDNA fragments corresponding to the C-terminal part of the human integrin subunits $\alpha 2$ (nt 3067–3591 as numbered by Takada and Hemler [32]), $\alpha 5$ (nt 2679–3176 as numbered by Argraves *et al.* [33]), and $\alpha \nu$ (nt 2655–3188) as numbered by Suzuki *et al.* [34]) were generated by polymerase chain reaction. The oligonucleotides used were: 5'-CCAGGATCCACTGGGGTGCAAAACAGACAAG ($\alpha 2$ sense primer), 5'-CGCGAATTCGCTACTGAGCTCTGTGGTCTC ($\alpha 2$ antisense primer), 5'-TAAGGATCCGGTTCCTGCACCACCAGCAA ($\alpha 5$ sense primer), 5'-CGAGAATTCGACTCAGGCATCAGAGGTGGC ($\alpha 5$ antisense primer), 5'-TCAGGATCCGAAAAGAATGACACGGTTGCC ($\alpha \nu$ sense primer), 5'-CTCGAATTCAGTTTCTGAGTTTCTTCACC ($\alpha \nu$ antisense primer). Total RNA from the human fibroblast cell line 1523 (Human Genetic Cell Mutant Repository, NJ) was used as template for synthesis of cDNA by M-MuLV reverse transcriptase (Perkin-Elmer Cetus) with the antisense oligonucleotides as primers. Each reaction was performed in 20 μl containing 1 μg RNA, 200 ng antisense oligonucleotide, 2.5 units of reverse transcriptase, 5 mM MgCl_2 , 1 mM dXTP, and 1 unit of RNase inhibitor. The primer was first heated to 68°C for 10 min together with the RNA, cooled to 4°C, and then added to the remaining ingredients. The reactions were incubated at 42°C for 1 h, followed by 5 min at 99°C to inactivate the enzyme. The subsequent amplification reactions were carried out after addition of the corresponding sense oligonucleotide in

a thermal cycler (Gene ATAQ controller, Kabi-Pharmacia AB) with Amp-litaq DNA polymerase (Perkin-Elmer Cetus) using 55°C for primer annealing and 72°C for polymer elongation. The cDNA products were cloned into pUC and sequenced to verify the products.

cDNA corresponding to the integrin subunit $\beta 1$ of rat was obtained by screening of a liver cDNA library in $\gamma\text{gt}11$ (Clontech) with an oligonucleotide derived from the chicken $\beta 1$ -sequence; 5'-GAATTTGCTAAATTTGAAAAGGAGAAAATGAATGCCAAGTGGGAC. The rat cDNA fragment used in this study corresponds to nt 1304–2601 in the mouse $\beta 1$ sequence published by Tominaga [35]. Oligonucleotides were synthesized on Gene Assembler Plus (Kabi-Pharmacia) from PAC amides.

Cell preparation. Bovine chondrocytes were isolated by collagenase digestion of articular cartilage from 4–6-month-old calves as described elsewhere [36]. Briefly, cartilage slices were digested by collagenase in EBSS (Earle's balanced salt solution) for 15–16 h at 37°C. The cells were filtered through a 100- μm nylon filter, washed three times in Dulbecco's modified phosphate-buffered saline (PBS), and used immediately after the isolation.

The human chondrosarcoma cell line (105KC) was established as described [37]. The cells were cultured in 40% Dulbecco's MEM, 40% MEM α with deoxyribose, 10% F12, and 10% fetal calf serum, pH 7.5, supplemented with 44 mM NaHCO_3 , 100 nM hydrocortisone, 100 ng/ml insulin, 25 $\mu\text{g}/\text{ml}$ ascorbic acid, 50 IU/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. To harvest cells prior to the experiments, the culture dish was washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and the cells were incubated with 0.05% trypsin and 0.5 mM EDTA in PBS ($-\text{Ca}^{2+}/\text{Mg}^{2+}$) for 5 min. Detached cells were suspended and washed once in Dulbecco's culture medium with 10% FCS and once in PBS. In some experiments the cells were treated with hyaluronidase for 30 min at 37°C and finally washed three times with PBS.

Surface labeling with ^{125}I . Freshly prepared cells ($5\text{--}20 \times 10^6$) were washed three times with PBS containing 1 mg/ml glucose and suspended in 1 ml of PBS-glucose. ^{125}I (1 mCi) was added to the cells together with 32 μl lactoperoxidase (1 mg/ml), 12 μl glucoseoxidase (a stock solution of 7.2 μl glucoseoxidase (1010 units/ml) + 2 ml PBS-glucose was prepared fresh), and the cells were kept on ice for 12–15 min. The cells were then washed once with Dulbecco's culture medium to stop the reaction and three times with PBS. The ^{125}I -labeled cells were then lysed in 1 ml of 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 100 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 1 mM PMSF, 1 mM MnCl_2 , and 1 mM MgCl_2 for 1 h on ice. The lysate was centrifuged for 30 min at 10,000 rpm and the pellet was discarded.

Coupling of collagen type II-Sepharose. Collagen type II was equilibrated in 0.4 M NaCl, 20 mM NaH_2PO_4 , pH 7.8, and mixed end-over-end with the washed CNBr-Sepharose overnight (10 mg protein/ml Sepharose). The Sepharose was then washed once with 0.2 M glycine, pH 8.0 (blocking buffer), incubated with the blocking buffer for 5 h at 14°C, and washed once with PBS and twice with 0.1 M acetic acid. The collagen-Sepharose was stored in 0.1 M acetic acid. The control-Sepharose was treated the same way except that protein was not added. All steps were performed at 4°C.

Affinity purification. Collagen type II-Sepharose (1 ml) and the control-Sepharose (0.5 ml) were equilibrated with at least 20 vol of 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 1 mM MnCl_2 , and 1 mM MgCl_2 . ^{125}I -labeled cell lysate was passed twice over the control-Sepharose and then incubated with the collagen-Sepharose end over end for 2–3 h. The collagen-Sepharose was washed (15 gel vol) with the equilibration buffer containing 75 mM NaCl and bound proteins were then eluted with 20 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 10×0.5 ml. The eluted protein peak was passed over a gel filtration column (PD10, Pharmacia, Sweden) equilibrated with TBS (Tris-buffered saline) containing 1% Triton X-100, 0.15 M NaCl, 0.1% BSA, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF, and then eluted with the same buffer. Samples of the affinity-purified proteins were then either precipitated by ethanol or

immunoprecipitated by antibodies followed by separation on 4–12% SDS–PAGE and visualized by autoradiography.

Immunoprecipitation. Radiolabeled proteins were immunoprecipitated from cell lysates and from the affinity-purified material. The cell lysates were precleared either with 100 μ g/ml rabbit IgG together with 100 μ l of protein A–Sepharose (1:1 slurry in PBS) or with 50 μ l of rabbit anti-mouse IgG conjugated to agarose. The samples were centrifuged for 5 min at 3000 rpm and the pellet was discarded. The pre-cleared cell lysate or affinity-purified samples were incubated overnight, end-over-end, with 5 μ l of monoclonal antibodies (β 1, α 1, α 2, α 3, α 5, or α v) or 50–100 μ g/ml of the polyclonal antibodies (β 1 and α 1) followed by 100 μ l protein A–Sepharose slurry or 50 μ l anti-mouse IgG–Sepharose for 45 min. The agarose beads were centrifuged for 5 min at 10,000 rpm, washed four times with 1% Triton X-100, 0.5 M NaCl, and 10 mM Tris–HCl, pH 7.4, and once with 10 mM Tris–HCl, pH 7.4. All steps were performed at 4°C. SDS–PAGE sample buffer (100 μ l) and 5% β -mercaptoethanol were added to the washed immunoprecipitate and the samples were boiled for 5 min with or without β -mercaptoethanol. The immunoprecipitated proteins were separated by 4–12% SDS–PAGE and visualized by autoradiography.

Mechanical stimulation. Bovine chondrocytes or human chondrosarcoma cells were grown in six-well silicon elastomer-bottomed culture plates with an amino surface (Flex I culture plates, Flexcell Corp., McKeesport, PA). In some experiments the dishes were coated with 10 μ g/ml of collagen type II in PBS. The mechanical stimulation experiments were performed with the Flexercell strain unit (Flexcell Corp.). The stress unit is a modification of the unit initially described by Baner *et al.* [38, 39] and consists of a computer-controlled vacuum unit and a baseplate to hold the culture dishes. Vacuum (around –18 kPa) is applied to the dishes via the baseplate, which is placed in a cell incubator. When a precise vacuum level is applied to the system, the culture plate bottoms are deformed to a known percentage elongation which is maximal at the edge and decreases to the center. The strain is translated to the cultured cells. When the vacuum is released the plate bottoms return to their original conformation. In the described experiments the culture plate bottoms were deformed to 24% maximal elongation (around average 10% increase in surface area) in a cyclic (2 s on, 2 s off) manner and the cells were stimulated for 1, 3, or 20 h.

Northern hybridization. At the end of the mechanical stimulation experiments the dishes were placed on ice and the cells were immediately detached with trypsin–EDTA. Cells from two six-well dishes (around 2×10^6) were combined and the mRNA was purified using mRNA Quick-prep (Pharmacia, Sweden). The mRNA was precipitated with ethanol and kept at –70°C until used. The mRNA was separated on a 1% agarose gel, transferred to nylon membranes, and immobilized by UV crosslinking. cDNA probes were 32 P-labeled with Random Primed DNA Labeling Kit (Boehringer-Mannheim). The filters were prehybridized for 2–4 h at 42°C in 5 \times SSC, 5 \times Denhart's solution, 1% SDS, 50 μ g/ml salmon sperm DNA, and 50% formamide, and then hybridized overnight at 42°C with the same solution containing 10% dextran sulphate and the specific probe (0.5–1 $\times 10^6$ cpm/ml). The filters were then washed 2 \times 10 min with 1 \times SSC and 2 \times 10 min with 0.1 \times SSC at room temperature and then 5 min or longer with 0.1 \times SSC at 68°C. Specifically bound cDNA probes were analyzed using a phosphorimage system (Fuji). The change in mRNA level of the integrins after 3 h of mechanical stimulation compared to the control is related to GAPDH on the same filter to adjust for any variation in amount of mRNA between the lanes. cDNA probes were stripped by washing the filters in 0.1% SDS, for 1 h at 80°C.

Statistics. Results in Fig. 4 are presented as means \pm SD and n = the number of individual experiments. Student's paired t test was used to determine statistical significance.

RESULTS

Affinity Chromatography of Collagen Type II-Binding Integrins on Chondrocytes

Collagen type II-binding proteins from Triton X-100 lysates of 125 I-labeled bovine primary chondrocytes

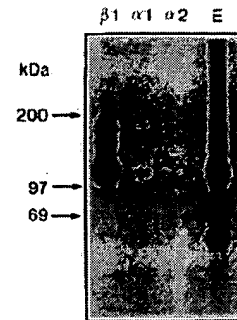


FIG. 1. Affinity purification and immunoprecipitation of collagen II-binding proteins from Triton X-100 lysates of 125 I-labeled bovine chondrocytes. The lanes show immunoprecipitation of the eluted proteins with polyclonal antibodies against β 1 and α 1, and a monoclonal antibody against α 2 (Gi19), respectively. The proteins eluted by EDTA from collagen type II–Sepharose is shown in lane E. The proteins were separated by SDS–PAGE (4–12%) under nonreduced conditions and visualized by autoradiography. For details see Materials and Methods.

were affinity purified on collagen type II–Sepharose. Figure 1 shows immunoprecipitation of proteins eluted from the collagen type II column and precipitated by polyclonal antibodies against β 1, α 1, and a monoclonal antibody against α 2 (Gi19), respectively. The results show that the integrins α 1 β 1 and α 2 β 1 were immunoprecipitated from the collagen type II–Sepharose eluate. A third collagen-binding β 1-associated α -subunit with M_r around 160 kDa (Fig. 1) was not identified by the polyclonal α 1-integrin or the monoclonal α 2-integrin (Gi19) antibodies. Polyclonal antibodies against human α 9-integrin (provided by Dr. Dean Sheppard), that react with bovine α 9-integrin, did not recognize this α -integrin subunit (data not shown), suggesting that the 160-kDa protein may represent a novel collagen type II-binding integrin. This is currently under investigation.

Affinity Purification of Collagen Type II-Binding Integrins on Chondrosarcoma Cells

We also affinity purified collagen type II-binding integrins from human chondrosarcoma cells. Immunoprecipitation of the material eluted from the collagen type II column showed that the integrins α 1 β 1 and α 2 β 1 are collagen type II-binding integrins on the chondrosarcoma cells (Fig. 2A). As in the case with primary chondrocytes, an unknown integrin slightly larger than α 2 was precipitated by the β 1-antibody. None of the monoclonal antibodies against the integrin subunits α 1, α 2, or α 3 were able to recognize this protein. As shown in Fig. 2B the integrin α 3 β 1 is present on human chondrosarcoma cells. Since this integrin is a possible collagen receptor we tested whether α 3 β 1 could bind to collagen type II but monoclonal antibodies

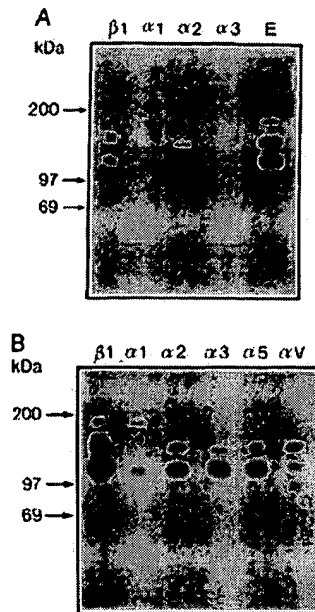


FIG. 2. (A) Affinity purification and immunoprecipitation of collagen II-binding proteins from Triton X-100 lysates of ^{125}I -labeled human chondrosarcoma cells. The lanes show immunoprecipitation of the collagen type II-binding proteins with monoclonal antibodies against the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$ (P1E6), and $\alpha 3$, respectively. The proteins eluted by EDTA from the collagen type II-Sepharose is shown in lane E. The proteins were separated by SDS-PAGE (4–12%) under reduced conditions and visualized by autoradiography. (B) Immunoprecipitation of ^{125}I -labeled human chondrosarcoma cell lysate by monoclonal antibodies against the human integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$ (P1E6), $\alpha 3$, $\alpha 5$, and αv . The immunoprecipitated proteins were separated by SDS-PAGE under nonreduced conditions and visualized by autoradiography. For details see Materials and Methods.

against integrin $\alpha 3$ failed to precipitate $\alpha 3\beta 1$ from material eluted from collagen type II-Sepharose (Fig. 2A).

Mechanical Stimulation of Chondrocytes and Expression of Matrix Components

Bovine chondrocytes were cultured for 1 week on flexible silicone membranes and then exposed to cyclic mechanical stress (15 cycles/min, 24% maximal elongation) by the Flexcell system. The mRNA was then purified and the mRNA expression was studied by Northern blotting. We found that cyclic mechanical stress for 3 h increased the mRNA levels of both collagen type II and aggrecan. Figure 3 shows the results from Northern blot experiments. The changes in mRNA expression were related to the GAPDH mRNA. We found in the same experiment that expression of $\beta 1$ -integrin was not changed after 3 h of cyclic mechanical stimulation. The available cDNA probes for α -integrin subunits crosshybridized too weakly to allow detection of the

bovine mRNAs. Therefore human chondrosarcoma cells were used in the subsequent experiments.

Mechanical Stimulation of Chondrosarcoma Cells and Integrin Expression

Chondrosarcoma cells were cultured (3–7 days) on uncoated Flexercell dishes (in the presence of serum) and exposed to mechanical stress for 1, 3, or 20 h (15 cycles/min, 24% maximal elongation). mRNA levels of the integrin subunits $\beta 1$, $\alpha 2$, $\alpha 5$, and αv in stimulated cells were compared with mRNA levels in nonstimulated cells by Northern hybridization. The results from seven different experiments are shown in Fig. 4 and the mean values are shown in the table in Fig. 4. We found in these experiments that the mean mRNA expression of $\alpha 5$ increased to a significant level after 3 h of mechanical stimulation while the increased mean expression of $\beta 1$, $\alpha 2$, and αv was not significant. As shown in Fig. 4, large variations were seen between different experiments. In two of the experiments shown in Fig. 4, $\alpha 2$ -integrin mRNA increased to a larger extent (around 50%) compared to the other experiments. In the same experiments mRNA expression of collagen type I was also increased (Fig. 4), which may indicate that $\alpha 2$ -integrin is upregulated when collagen synthesis increases. We were not able to detect synthesis of collagen type II in the chondrosarcoma cells. In some of the experiments shown in Fig. 4, the cells were exposed to mechanical stress for 20 h. The mRNA expression at this time point appeared similar compared to expression after 3 h. The 20-h results were not included in the statistical analysis since this time point was absent in some of the experiments.

In other experiments human chondrosarcoma cells were cultured on collagen type II-coated Flexercell dishes for 2 days and exposed to cyclic mechanical stimulation (15 cycles/min, 24% maximal elongation) for 3 h (Fig. 5). We found in these experiments that mechanical stimulation increased the mRNA expression of the $\alpha 2$ -integrin subunit but not the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 5$, and only to some extent αv . These results indicate that $\alpha 2\beta 1$ -integrin is a collagen type II-binding integrin on chondrosarcoma cells that is upregulated in response to mechanical stimulation. In contrast to the experiments where chondrosarcoma cells were cultured on uncoated dishes (Fig. 4) the mRNA expression of $\alpha 2$ -integrin was higher than the expression of $\alpha 5$ -integrin, which may indicate that matrix molecules can modulate the integrin response to mechanical stress.

DISCUSSION

We have found in the present investigation that the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are collagen type II-binding integrins on both primary bovine chondrocytes and hu-

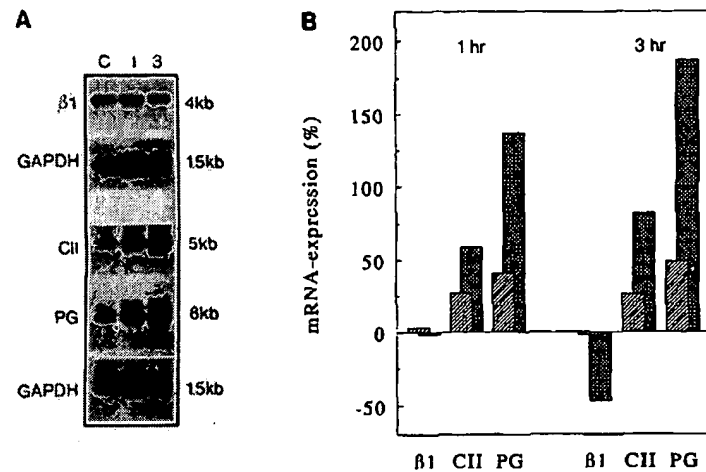


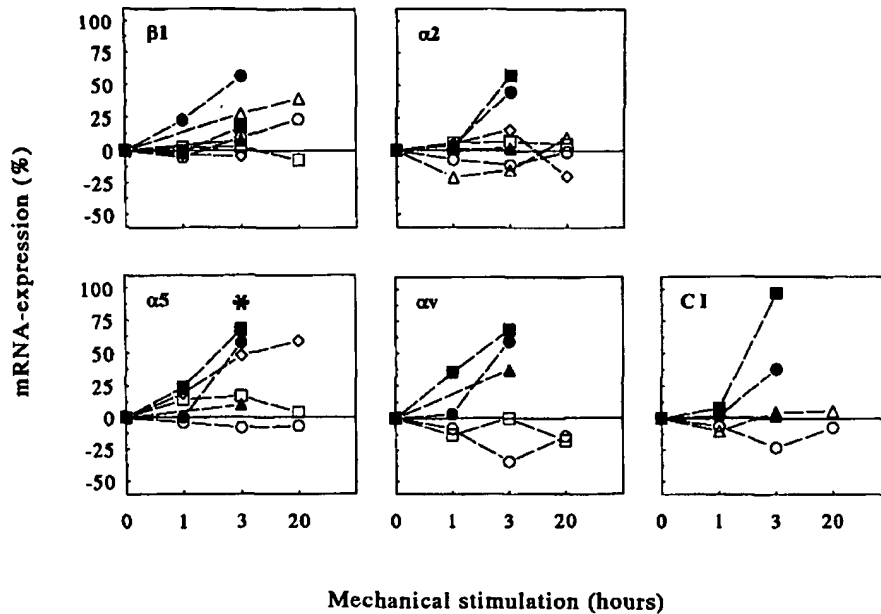
FIG. 3. Expression of $\beta 1$ -integrin ($\beta 1$), collagen type II (CII), and the proteoglycan aggrecan (PG) during mechanical stimulation of bovine chondrocytes. A shows pictures of Northern blots from one experiment. The mRNA was divided on two filters: one was hybridized with ^{32}P -labeled cDNA corresponding to $\beta 1$ -integrin followed by GAPDH cDNA and the other with ^{32}P -labeled cDNA corresponding to collagen type II or aggrecan followed by GAPDH cDNA. The lanes show hybridized mRNA from control cells (C) and from cells exposed to mechanical stress for 1 (1) or 3 h (3). The radioactivity of the mRNA bands was analyzed and the mRNA levels of $\beta 1$, CII, and PG in relation to the mRNA level of GAPDH were determined. In B, the change in $\beta 1$ -integrin, collagen type II, or proteoglycan mRNA after 1 or 3 h of mechanical stimulation, compared to the control, is shown. Results from two individual experiments are presented. For details see Materials and Methods.

man chondrosarcoma cells (105KC). Integrin $\alpha 1\beta 1$ has earlier been shown to interact with collagen types I, IV, and VI, and laminin (reviewed by Kühn and Eble [40]) but this is to our knowledge the first report demonstrating that $\alpha 1\beta 1$ is a receptor for collagen type II. Integrin $\alpha 2\beta 1$ has been shown to be a receptor for collagen type II on fetal chondrocytes while $\alpha 1\beta 1$ appear not to be present at this stage [23, 41]. This may indicate that chondrocytes switch collagen type II receptors during their development. In addition to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ we have found a collagen type II-binding $\beta 1$ -integrin with an α -subunit slightly larger than $\alpha 2$. This integrin-subunit with an M_r around 160 kDa is present on both bovine chondrocytes and human chondrosarcoma cells but we have not been able to identify it with any of the antibodies against the integrin subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 9$. The possibility that this is a novel α -subunit is currently under investigation. The integrin $\alpha 3\beta 1$, on a lung carcinoma cell-line, has been shown to interact with collagen type I, although with low affinity [22]. On the other hand Gullberg *et al.* [18] reported that $\alpha 3\beta 1$ on rat fibroblasts did not bind to collagen type I. We show in this study that $\alpha 3\beta 1$ on human chondrosarcoma cells appeared not to interact with collagen type II as judged from affinity chromatography (Fig. 2A). Similar results have been reported by Tuckwell *et al.* [42] using a different chondrosarcoma cell line (HCS-2/8). The integrin $\alpha 3\beta 1$ is also present on primary chondrocytes [24] but its ligand in cartilage is not known.

The cartilage tissue depends on mechanical load for

normal function but the mechanisms are not understood. The chondrocyte matrix receptors, including the collagen type II receptors, very likely play an important role as sensors of tension in the cartilage matrix network and mediate mechanical signals to the chondrocyte. It is known from other studies that cyclic mechanical stimulation of cartilage increases matrix production while a static stimulation, on the other hand, decreases the production of matrix components [9]. In the present investigation, cells were cultured on flexible-bottomed culture dishes and exposed to mechanical stress by the Flexercell technique. A negative pressure stretches the flexible membrane and the strain is transferred to the cells. The strain is variable over the membrane with the maximal strain close to the edge and minimal strain in the middle of the dish. In addition, the edge of the dish is not flexible (around 40% of the total area) and part of the cells in the dish are therefore not stretched. Thus, in one dish both nonstimulated cells and cells that are exposed to variable strain will contribute to the experiment and changes due to mechanical stress may therefore appear low. It may be argued that the chondrocytes are compressed and not stretched during loading of intact cartilage but it is very likely that collagen and other matrix components of the extracellular matrix network, that are linked to the chondrocytes, indeed stretch the cells during compression of cartilage. With this technique we can focus on the direct effect of mechanical stretching of the cells.

Dynamic stimulation of bovine chondrocytes was found to increase the mRNA expression of the cartilage



	$\beta 1$	$\alpha 2$	$\alpha 5$	αv	C1
1 hr	4 ± 10 $n=6$	-2 ± 10 $n=6$	11 ± 12 $n=5$	11 ± 25 $n=4$	-2 ± 8 $n=4$
3 hrs	16 ± 21 $n=7$	15 ± 28 $n=7$	32 ± 30 $n=6$	28 ± 39 $n=5$	24 ± 46 $n=5$

FIG. 4. Expression of integrin mRNA in human chondrosarcoma cells during mechanical stimulation. The cells were cultured on uncoated dishes, in the presence of serum, and the mRNA was extracted from control cells and after 1, 3, or 20 h of mechanical stimulation. The mRNA was hybridized with ^{32}P -labeled cDNA probes against the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 5$, αv , collagen type I, and GAPDH. The radioactivity of the hybridized mRNA bands from Northern blots was analyzed and the mRNA level of the integrin subunits and collagen type I was related to the mRNA level of GAPDH. The figure shows the change in integrin and collagen mRNA after mechanical stimulation, compared to the control, in seven individual experiments. The table shows the mean mRNA changes at 1 and 3 h \pm SD and n represents number of experiments. Only the $\alpha 5$ -integrin subunit was found to increase to a significant level ($P = 0.047$) according to Student's paired t test.

matrix proteins collagen type II and proteoglycan with similar results after 1 and 3 h. The synthesis is not just an overall increase in mRNA since the changes in collagen type II and proteoglycan were related to the GAPDH mRNA in the same experiments. The finding that collagen and proteoglycan synthesis increases during cyclic mechanical stimulation is in agreement with earlier studies where it was found that mechanical stimulation of cartilage explants increased the production of proteoglycans and proteins [9–11]. We have not investigated whether mechanical stress increased the turnover of these proteins.

Since collagen type II increased during dynamic stress it was of great interest to study the expression of the collagen type II-binding integrins. We were able to study mRNA expression of $\beta 1$ -integrin on bovine chondrocytes and we found, in the same experiments where matrix synthesis was studied, that the $\beta 1$ -integrin subunit was not changed or in some cases decreased in response to mechanical stress. It is possible

that the $\beta 1$ -integrin is not synthesized but rather redistributed during reorganization of the cell-matrix contacts when the chondrocytes were exposed to mechanical stimulation. We were not able to study the effect of mechanical stimulation on expression of $\alpha 1$ - and $\alpha 2$ -integrin subunits since available cDNA probes hybridized too poorly with the bovine $\alpha 1$ - and $\alpha 2$ -mRNA. To study the effect of mechanical stimulation on these integrin subunits we used human chondrosarcoma cells. These cells synthesized aggrecan and collagen type I instead of collagen type II and cannot be defined as differentiated chondrocytes. However, we found in affinity chromatography experiments that the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were present on human chondrosarcoma cells and that these integrins bound to immobilized collagen type II (Fig. 2) which makes these cells useful in the mechanical stimulation study.

Mechanical stimulation of chondrosarcoma cells cultured on collagen type II-coated dishes increased the mRNA expression of $\alpha 2$ - but not $\alpha 1$ -integrin (Fig. 5),

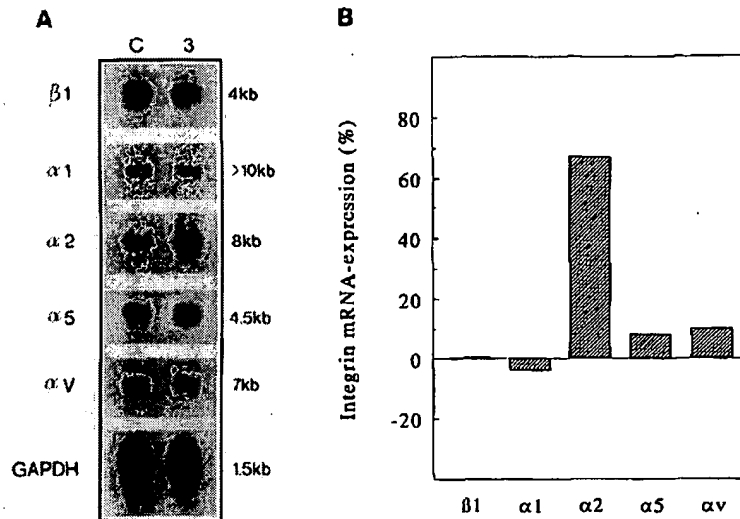


FIG. 5. Expression of integrin mRNA in human chondrosarcoma cells during mechanical stimulation. A shows phosphorimage pictures of Northern blots from one experiment. The cells were cultured on collagen type II-coated dishes and the mRNA was extracted from control cells (C) and after 3 h of mechanical stimulation (3). The mRNA was hybridized with ^{32}P -labeled cDNA probes corresponding to the integrin subunits $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 5$, and αv , and GAPDH. The same filter was used for all cDNA probes. The radioactivity of the hybridized mRNA bands was analyzed and the mRNA levels of integrin subunits were related to GAPDH mRNA. B shows the change in integrin mRNA level after mechanical stimulation, compared to the control. Results from one of two parallel experiments are shown. For details see Materials and Methods.

indicating that $\alpha 2$ -integrin subunit may be involved in reorganizing the cell–collagen contacts. This is in agreement with the report by Klein *et al.* [43] where it is shown that integrin $\alpha 2\beta 1$ but not $\alpha 1\beta 1$ or $\alpha 3\beta 1$ is upregulated when fibroblasts are seeded into contracting type I collagen gels. We also found that expression of collagen type I mRNA increased during mechanical stimulation in those experiments where expression of $\alpha 2$ -integrin subunit increased to a larger extent (Fig. 4), which may indicate that $\alpha 2\beta 1$ -integrin is involved in synthesis and organization of collagen molecules.

The response in integrin expression appeared to be different when the cells were cultured on noncoated or collagen type II-coated dishes, which may indicate that matrix molecules can modulate the mechanical signal. We found that only the expression of $\alpha 5$ -integrin increased to a significant level when the cells were cultured on uncoated dishes (in the presence of serum) while especially the $\alpha 2$ -integrin subunit increased when the cells were cultured on collagen type II dishes. When the cells were cultured on uncoated dishes, integrins such as $\alpha 5\beta 1$ and/or αv in combination with $\beta 1$, $\beta 3$, or $\beta 5$ likely mediated the adhesion to serum components as fibronectin and vitronectin. In some of the experiments shown in Fig. 4, the cells were exposed to mechanical stimulation for 20 h and the mRNA expression was similar at this time point to mRNA expression at 3 h. To compare changes in integrin expression on the mRNA level with changes on the protein level we

analyzed expression of the integrin subunits $\beta 1$, $\alpha 2$, $\alpha 5$, and αv by flow cytometry in parallel with the experiments shown in Fig. 5. Mechanical stimulation for 3 h did not change the number of cell surface integrins to a significant level. It is likely that longer periods of mechanical stimulation is required to increase integrin expression on the protein level.

We have found variations between experiments that we at this point cannot explain. It is possible that cell density is one of the variables. Cell–cell contacts in confluent cultures may reduce the tension in the cells–matrix interactions and thereby affect expression of integrins. Serum factors may be other variables and to minimize variations due to these factors the cells were fed with fresh serum-containing medium the day before the experiment. The changes in mRNA shown in the figures have been related to the GAPDH mRNA. It is possible that mechanical stimulation also increases the GAPDH mRNA to some extent and small changes in integrin mRNA may therefore not be seen. In some of the experiments in which the integrin changes were related to both GAPDH and β -actin the results appeared similar (data not shown).

The results presented in this investigation will contribute to understanding the mechanisms involved in communication between chondrocytes and cartilage extracellular matrix. Although stretching of chondrocytes in monolayer cultures is a simplified model of loading intact cartilage, the use of such techniques will enable

us to identify signal pathways involved in mechanotransduction.

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protein passed through the column and was judged $\geq 98\%$ pure on a Coomassie-blue-stained SDS/polyacrylamide gel following concentration by vacuum dialysis in a collodion bag (Schleicher & Schuell). Selenomethionyl-substituted (SeMet) protein was prepared by using the methionine-auxotrophic strain 834(DE3) as described (20) and was purified in the same manner as the native protein.

Crystallization. Crystals were grown from hanging drops by the method of vapor diffusion. One microliter of 5 mM $\text{MnCl}_2/5$ mM 2-mercaptoethanol containing CD11a-I at 15 mg/ml was mixed with 1 μl of a reservoir solution of 14–20% (wt/vol) PEG 3350/100 mM $\text{MnCl}_2/50$ mM sodium acetate, pH 5.2, and equilibrated over the reservoir solution. Microseeding proved useful for both native and SeMet protein. Diffraction-quality crystals of SeMet protein were obtained only when the crystallization trials were carried out with degassed buffers in an anaerobic chamber. Crystals typically grew to a final size of 0.2 mm \times 0.1 mm \times 0.1 mm over 3–7 days. Most crystals were in space group C2 with unit cell dimensions $a = 131.13$ Å, $b = 45.45$ Å, $c = 66.13$ Å, and $\beta = 99.8^\circ$. The largest SeMet crystal was in space group P222 with unit cell dimensions $a = 66.12$ Å, $b = 129.22$ Å, and $c = 45.46$ Å. This crystal was used for multiwavelength anomalous diffraction (MAD) experiments.

Data Collection and Processing. All data were collected from crystals soaked for at least 15 min in reservoir buffer containing 20% (wt/vol) PEG and 10% (vol/vol) ethylene glycol and flash-frozen in a gaseous nitrogen stream at -180°C . Diffraction data were collected from native C2 and SeMet P222 crystal forms with an R-axis IIC detector and Cu K α radiation from a Rigaku RU200 rotating anode. MAD data were collected from a single SeMet P222 crystal that was transported frozen to the X4A beamline of the National Synchrotron Light Source at Brookhaven National Laboratory. MAD data were collected with Fuji HR-5 phosphor imaging plates. Two-degree oscillations at ϕ and $\phi + 180^\circ$ were collected with no overlap for each oscillation range at each wavelength. All diffraction images were processed with the program DENZO and scaled with the program SCALEPACK (21). (I^+) and (I^-) were used for MAD phase determination, and partially recorded reflections were used in all cases. Diffraction data from different wavelengths were scaled with WVLSCAL, and F_A values and optimal f' and f'' values were calculated with MADLSQ (22).

Structure Determination. Four selenium sites were deduced from inspection of F_A Patterson and difference Fourier maps, and these sites were confirmed with the program HEAVY. Phase determinations were made with the program MLPHARE, and solvent flattening and histogram matching were performed with the program DM (23). A complete atomic model for residues Asn-129 to Tyr-307 was readily built into 2.6-Å electron density maps by using the program O (24). The placement of this model in the unit cell indicated that the asymmetric unit must contain an additional molecule. All four selenium sites were accounted for by the first model, however, and no interpretable density existed for a second molecule. The initial model was thus used as a search model for molecular replacement with the C2 crystal form. Two solutions were found in the asymmetric unit of the C2 crystal form by use of X-PLOR (25), and all refinement was carried out in this crystal form. Several rounds of simulated annealing and/or Powell minimization with X-PLOR alternated with model building with O have produced the current atomic model for CD11a-I. This model consists of residues Gly-128 to Ile-309 for one molecule in the asymmetric unit, Gly-128 to Glu-310 for the other molecule (2941 nonhydrogen protein atoms), 2 manganese ions, 2 chloride ions, and 312 water molecules. A chloride ion has been modeled in one of the manganese coordination sites, as the distance to the manganese at this site (2.55 Å) is more compatible with chloride than with water, difference Fourier maps indicate a strong scatterer at this

position, the charge on a chloride would neutralize the metal coordination sphere, and chloride is the only monoatomic anion added to the buffer. The rms difference in position for all main-chain atoms following superposition of the independent molecules in the asymmetric unit is 0.17 Å. Refinement statistics and stereochemical parameters for this model are shown in Tables 1–3.

RESULTS

The CD11a I-domain structure consists of a five-stranded parallel β -sheet core surrounded on both faces by α -helices (Fig. 1). A short antiparallel strand occurs on one edge of this sheet. The position of secondary structural elements in the CD11a sequence is shown in Fig. 2. This structure is very similar to that of flavodoxin (26) and members of the dinucleotide-binding family of proteins (27). The topology of the CD11a I-domain is essentially identical to that reported for the CD11b I-domain (17), with the exception that a 7-aa deletion between β -strands 3 and 4 ($\beta 3$ and $\beta 4$) in CD11a relative to CD11b results in the shortening of α -helix 5 ($\alpha 5$) to a single turn of 3_{10} -helix. Single turns of 3_{10} -helix are also found at the end of $\alpha 6$ (Leu-274 to Phe-277), between $\alpha 4$ and $\beta 3$ (Arg-221 to Gly-225; not shown in Fig. 1), and between $\beta 4$ and $\alpha 6$ (Lys-263 to Gln-266). An unusual turn is found between $\beta 2$ and $\beta 2'$ that contains a hydrogen bond from the amide of Ser-175 to the carbonyl of Ser-177. This turn appears similar to a γ turn, with the difference that the direction of the main-chain hydrogen bond is reversed. The only residues identified to be in highly strained regions of a Ramachandran plot by the program PROCHECK (28) are Ser-175, Leu-204, and Leu-205. Electron density for each of these residues is persuasive, and stabilizing hydrogen bonds exist in all cases.

The residues whose side chains coordinate the metal ion in the CD11a and CD11b I-domains are completely conserved [the so-called MIDAS motif (17)], but the manner in which the metal is coordinated differs slightly. As shown in Fig. 3 for the CD11a I-domain, the side chains of Ser-139, Ser-141, and Asp-239 directly coordinate the bound metal while the side chains of Thr-206 and Asp-137 are hydrogen-bonded to coordinating water molecules. In the CD11b I-domain the Thr-206 homologue directly coordinates the metal while the Asp-239 equivalent is hydrogen-bonded to a coordinated water molecule. Comparison of the CD11a I-domain structure with the published figures of the CD11b I-domain suggests that these differences in coordination may be achieved by a rotation of the coordinated metal ion. Other than affecting the interactions with Thr-206 and Asp-239, the principal effects of such a rotation would be to alter the orientation of the most solvent-exposed metal coordination position. The differences in metal coordination seen in the CD11a and CD11b structures may thus reflect a difference between I-domains with protein-liganded and solvent-exposed metal-binding sites. There seems little reason to believe any significant structural or functional differences exist between Mn^{2+} - and Mg^{2+} -bound forms of I-domains, and any variations in the physiological effects of these ions seems likely to be due to their differing affinities for I-domains (15).

Another notable difference between the CD11a and CD11b I-domain structures appears to be the disposition of $\alpha 7$ relative to the body of the I-domain. The extent of $\alpha 7$ is identical in CD11a and CD11b, and comparison of the $\alpha 7$ sequences in the two molecules reveals identical residues at 50% of the positions and residues conserved in type at $>90\%$ of the positions. Despite these similarities, $\alpha 7$ appears to have undergone significant rigid-body motion when the structure of the CD11a and CD11b I-domains are compared. A likely source for this motion is found by examination of intermolecular contacts for CD11a. $\alpha 7$ forms the C terminus of I-domains, and the C termini of the two CD11a I-domains in the asymmetric unit of

Crystal structure of the I-domain from the CD11a/CD18 (LFA-1, $\alpha_L\beta_2$) integrin

(metal-binding site/protein structure/cell-cell adhesion/von Willebrand disease)

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ABSTRACT We report the 1.8-Å crystal structure of the CD11a I-domain with bound manganese ion. The CD11a I-domain contains binding sites for intercellular adhesion molecules 1 and 3 and can exist in both low- and high-affinity states. The metal-bound form reported here is likely to represent a high-affinity state. The CD11a I-domain structure reveals a strained hydrophobic ridge adjacent to the bound metal ion that may serve as a ligand-binding surface and is likely to rearrange in the absence of bound metal ion. The CD11a I-domain is homologous to domains found in von Willebrand factor, and mapping of mutations found in types 2a and 2b von Willebrand disease onto this structure allows consideration of the molecular basis of these forms of the disease.

CD11a/CD18 (LFA-1, $\alpha_L\beta_2$) is a member of the integrin family of cell surface receptors. Integrins consist of a 120- to 180-kDa α subunit (CD11a in this case) and a 90- to 110-kDa β subunit (CD18 or β_2 in this case) that are noncovalently associated single-pass transmembrane proteins. The bulk of each integrin subunit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or counterreceptors on the surface of apposed cells (1). CD11a/CD18 is expressed on all leukocytes and mediates adhesion to a variety of cell types that express one or more of the CD11a/CD18 ligands intercellular adhesion molecule 1 (ICAM-1), ICAM-2, and ICAM-3. The processes mediated by CD11a/CD18 interactions include adhesion to the endothelium and extravasation at sites of inflammation and adherence of activated T cells to target cells (2). A subset of integrin α chains, including CD11a, contain an inserted domain (I-domain) of ~ 190 aa that is homologous to the family of von Willebrand Factor (vWF) A-type domains (3, 4). The I-domain has been associated with ligand binding in each of the I-domain-containing integrins (5-11).

Many integrins do not normally exhibit high affinity for ligands and must become activated for an interaction with ligand to be observed (1, 12, 13). The conversion to a high-affinity state is typically transiently induced after appropriate physiological stimuli to the integrin-bearing cell. The production of monoclonal antibodies that specifically recognize the active state of integrins and the observation of altered biophysical properties following ligand binding have led to the belief that a conformational change accompanies the transition to the high-affinity state in integrins (1). The mapping of both ligand-binding and active-state antibody epitopes to the I-domains of I-domain-containing integrins indicates that any activation-dependent conformational change is likely to involve the I-domain (7, 8, 14). The presence of a conserved divalent cation-binding site in I-domains and the requirement of divalent cations for ligand binding indicate that the metal-bound form of the I-domain is likely to represent a high-affinity state (6, 15). A regulated affinity for ligand is also

observed for the interaction between the vWF A domains and the platelet receptor GPIb (16), suggesting that conformational regulation of affinity for ligand may be a general feature of this type of domain.

We undertook an x-ray crystallographic study of the CD11a I-domain to investigate the structural features of affinity modulation and ligand binding in this domain and to provide a basis for modeling homologous domains in other proteins. Recently, the crystal structure of the I-domain from CD11b with bound magnesium was reported (17). The CD11b I-domain amino acid sequence matches the CD11a sequence at 33% of the positions, and the CD11b structure showed these domains to possess a flavodoxin-like tertiary structure and allowed identification of a set of conserved residues involved in coordinating the metal ion. We report here the refined 1.8-Å crystal structure of the CD11a I-domain with bound manganese ion.[†] A strained hydrophobic ridge adjacent to the bound metal ion is seen that may be involved in ligand binding and that is likely to undergo rearrangement in the absence of bound metal. We have also mapped von Willebrand disease (vWD)-causing mutations found in vWF A domains onto the CD11a I-domain structure and consider molecular mechanisms that may underlie different forms of vWD.

MATERIALS AND METHODS

Expression and Purification of the CD11a I-Domain. A DNA fragment encoding residues Cys-125 to Gly-311 of human CD11a (CD11a-I) was amplified by reverse transcription-PCR from the monocytic cell line THP-1 (18). The DNA sequence of this fragment was determined and was found to agree with published sequences (3) with the exception of a T \rightarrow C mutation that results in an Arg at position 189 instead of a Trp. Since multiple independent inserts contained this mutation and the proofreading *Pfu* polymerase was used, this mutation may represent an isoform. The CD11a gene fragment was subcloned into the pET11c expression vector and transformed into *Escherichia coli* BL21(DE3) and 834(DE3) cells (19). Three to 4 hr after induction of logarithmic-phase cells with isopropyl β -D-thiogalactopyranoside, the cells were harvested and lysed by sonication. The CD11a-I protein was found in the insoluble fraction of the lysate and resolubilized in 7 M urea/5 mM MnCl_2 /10 mM Tris, pH 7.5/40 mM 2-mercaptoethanol. The resolubilized pellet was dialyzed in successive steps over 2 days into 5 mM MnCl_2 /10 mM Tris, pH 7.5/5 mM 2-mercaptoethanol. A precipitate was removed by centrifugation, and the supernatant was concentrated by ultrafiltration and loaded onto a Pharmacia Mono-Q column. The CD11a-I

Abbreviations: ICAM, intercellular adhesion molecule; vWF, von Willebrand factor; vWD, von Willebrand disease; SeMet, selenomethionyl-substituted; MAD, multiwavelength anomalous diffraction.

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[†]The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1LFA, 1LFA-SF).

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Table 1. MAD structure-factor ratios and anomalous scattering factors

λ , Å	Structure-factor ratio*				Anom.†	
	0.9871	0.9793	0.9790	0.9686	$f'(e)$	$f''(e)$
0.9871	0.032	0.045	0.034	0.032	-4.03	0.51
0.9793		0.047	0.032	0.049	-9.77	3.95
0.9791			0.058	0.037	-7.09	5.55
0.9686				0.048	-3.59	4.00

*Ratio = $(\text{rms } (\Delta I)/I)/(\text{rms } (I)/I)$, where ΔI is the Bijvoet difference at one wavelength (diagonal elements) or the dispersive difference between two wavelengths (off-diagonal elements). Centric data were merged to a single value and are thus not shown. The Bijvoet difference at $\lambda 1$ may be taken instead as an upper limit of the noise of the anomalous signals.

†Anomalous components of the selenium scattering factors as a function of wavelength as determined by MADLSQ (22).

Table 2. Data collection statistics

Crystal	λ , Å	Resolution, Å	No. of reflections	Completeness, %	R_{sym}	$I/\sigma(I)$
P222	0.9871	30–2.6	10,170	80.5	6.3	19.1
P222	0.9793	30–2.6	10,207	80.8	6.7	19.6
P222	0.9790	30–2.6	10,105	79.9	7.3	19.0
P222	0.9686	30–2.6	10,162	80.5	6.7	18.1
C2	1.54	30–2.1	21,810	95.5	3.8	21.6
C2	1.54	30–1.8	27,543	76.0	4.0	20.3

R_{sym} and completeness values were calculated by considering Bijvoet's equivalent. $R_{\text{sym}} = 100 \times \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_i I_i(h)$.

Table 3. Refinement and stereochemical statistics for C2 crystal form

R value	0.190 ($F > 2\sigma$, 6.0–1.8 Å), 0.193 (all F , 6.0–1.8 Å)
R free	0.251 ($F > 2\sigma$, 6.0–1.8 Å), 0.256 (all F , 6.0–1.8 Å)
rms deviation	
Bonds, Å	0.009
Angles	1.9°
B value	1.2 / 1.3 (bonds/angles, main chain)
	2.3 / 2.7 (bonds/angles, side chain)
Avg. B, Å ²	14.9 (protein), 26.9 (solvent)

A subset of the data (5%) was excluded from refinement and used for the free R-value calculation (25). All data for which $|F| > 2\sigma$ were used in the refinement. R value = $\sum |F_o| - |F_c| / \sum |F_o|$.

the crystal structure reported here interact in such a way that residues 305–308 intercalate between $\alpha 7$ and the body of the molecule. As the CD11a I-domain is normally embedded in the much larger CD11a polypeptide, this interaction is almost certainly an artifact of crystal packing. $\alpha 7$ appears nonetheless quite labile, and this lability may prove a contributing factor to any conformational changes associated with the activation of CD11a/CD18 for ligand. A more complete consideration of structural differences between the CD11a and CD11b I-domains awaits direct comparison of atomic coordinates.

DISCUSSION

We have determined the high-resolution crystal structure of the I-domain of CD11a with bound manganese ion. The CD11a I-domain has been shown to contain a binding site for the CD11a/CD18 ligands ICAM-1 and ICAM-3 (7), but some manner of activation is required for this binding to be observed. This activation is believed to be accompanied by a conformational change in the integrin, most likely involving the I-domain. The ability of manganese ion to induce the active state and the requirement of divalent cations for ligand binding indicate that the manganese-bound form of the CD11a I-domain is likely to represent a high-affinity state of the molecule.



FIG. 1. Ribbon diagram of the CD11a I-domain. The manganese ion is shown in purple and the side chains of Ser-139, Ser-141, and Asp-239 are shown in green with red oxygen atoms. The N and C termini, the β -strands (yellow arrows), and the α -helical segments are labeled. This figure was made with the program MOLSCRIPT (26).

The presence in the CD11a I-domain structure of a strained hydrophobic ridge with main-chain contacts to the loop primarily responsible for coordinating the metal ion is consistent with both the existence of a conformational change upon activation and that the metal-bound state represents this active form. As shown in Fig. 3, Leu-203, Leu-204, Leu-205, and Met-140 form a solvent-exposed hydrophobic ridge adjacent to the metal-binding site. In addition to the energetic cost of exposing these hydrophobic residues, the main-chain torsion angles of Leu-204 ($\phi = -126$, $\psi = -138$) and Leu-205 ($\phi = -82$, $\psi = -159$) exist in highly unfavorable regions of ϕ , ψ space. These strained conformations are stabilized by the presence of the manganese ion through multiple main-chain hydrogen bonds between these residues and the metal-coordinating loop (residues 137–141). The presence of main-chain contacts between the hydrophobic ridge and the metal-coordinating loop suggests that this conformation is potentially independent of the amino acid sequence and may be a general feature of metal-bound states of I-domains. Metal coordination is also likely to affect the conformation of leucines 203–205 through their immediate successor in amino acid sequence, Thr-206, which forms hydrogen bonds with a metal-coordinated water in the CD11a structure and directly coordinates the metal in the CD11b structure (17). The conformation of the hydrophobic ridge is also stabilized through multiple hydrogen bonds to the unusual β -turn (residues 174–176) that contains the only other residue in the CD11a I-domain, Ser-174, with unfavorable main-chain torsion angles ($\phi = -155$, $\psi = -108$). Thus, two energetically strained loops exist whose conformations are stabilized directly and indirectly by the metal-coordinating loop. Significant rearrangements in these regions seem likely upon removal of the metal ion and may underlie the conformational change believed to occur during the transition between high- and low-affinity states of CD11a/CD18. The presence of this exposed hydrophobic

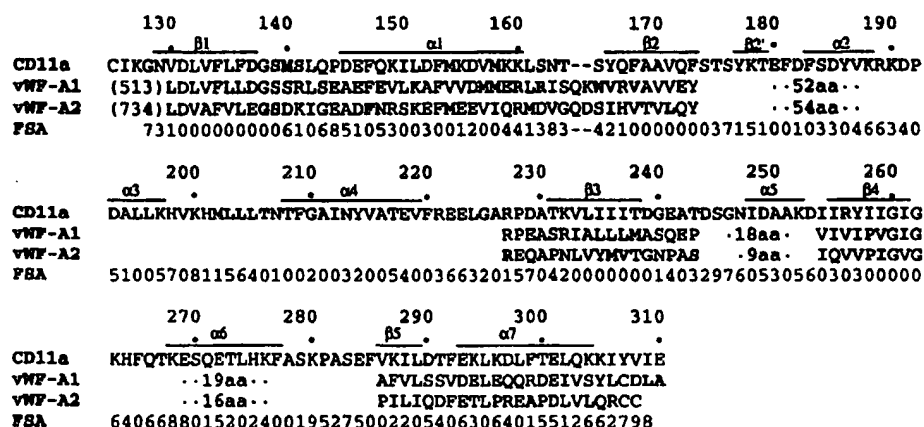


Fig. 2. Sequence alignment of the CD11a I-domain and vWF A domains. The amino acid sequence of the CD11a I-domain is shown with residues involved in coordinating the manganese colored purple and exposed hydrophobic residues near the manganese-binding site colored green. The secondary-structure assignments obtained by using the algorithm of Kabsch and Sander (27) as implemented in PROCHECK (28) are indicated. Sites of type 2b vWD-causing mutations in the vWF A1 domain are colored red, whereas sites of type 2a vWD-causing mutations in the vWF A2 domain are colored blue. Fractional solvent accessibility (FSA) is shown in green for each residue in the CD11a structure. The FSA is the ratio of the solvent-accessible surface area of a residue in a Gly-Xaa-Gly tripeptide vs. that in the CD11a I-domain structure. A value of 0 represents a value from 0.00 to 0.09, 1 represents 0.10 to 0.19, and so on.

region does not lead to aggregation of this domain as judged by dynamic light scattering, however, and this domain is soluble to at least 15 mg/ml in aqueous solution.

The exposed hydrophobic ridge provides a plausible binding site for the ICAMs. Considerable free energy could be gained by burying these residues at an appropriate intermolecular interface, and several hydrophobic residues in ICAM-1 and ICAM-3 exist in positions likely to be solvent exposed and near residues that affect the CD11a/CD18-ICAM interaction when mutated (e.g., residues 44-46 and 66 in ICAM-3 and residues 42-44 and 64 in ICAM-1) (29). The proximity of this hydrophobic ridge to the metal-binding site makes involvement of these residues in ligand binding consistent with speculation that an exposed coordination site on the metal ion may coordinate an acidic side chain from a ligand molecule (17). Only one position in the exposed hydrophobic ridge identified in CD11a remains hydrophobic for both CD11b and CD11c, suggesting that a high degree of hydrophobicity in this region is not a general requirement for I-domain activation and may be specific to the interactions of CD11a. The probable presence of I-domain-like structures in integrin β subunits (17), however, indicates that structural rearrangements in I-domain-like structures may prove a general feature of affinity modulation by integrins.

The homology between the CD11a I-domain and A-type domains from vWF allows mapping of the position of vWD causing mutations in the A1 and A2 domain of vWF onto the CD11a I-domain structure. vWD is an hereditary bleeding disorder of man that results from abnormalities of vWF (16). An alignment of CD11a and vWF amino acid sequences is shown in Fig. 2. By using the fractional solvent accessibility of the amino acids in CD11a structure as a guide, the hydrophobic β -strands of these domains could be confidently aligned. Gaps in the sequence alignment were left in less certain regions. All but two of the type 2b vWD-causing mutations catalogued in ref. 30 are shown in red in Fig. 4 and can be seen to cluster in a single area of the molecule. The two mutations not shown could not be confidently placed in the CD11a sequence, but it is likely that they map to this area also. The majority of these mutations occur at solvent-exposed sites (Fig. 4). The affinity of vWF for its platelet receptor, GPIb, is normally not observable, and an activation event is required for a high-affinity interaction. Mutations causing type 2b vWD result in a gain-

of-function phenotype in which vWF has a constantly high affinity for GPIb. Mutations at several positions of a ligand-receptor binding site are more likely to decrease the affinity of the interaction than increase it as is seen in type 2b vWD, and mutations of solvent-exposed residues are unlikely to alter the global structure of the molecule. We therefore speculate that the vWF A1 mutations cause type 2b vWD by interfering with an interaction of vWF A1 domains that normally inhibits GPIb binding (either sterically or by inducing an inactive conformation) and that the site of these mutations does not represent the GPIb-binding site. A scanning mutagenesis study of vWF led to a similar conclusion (31).

Sites of type 2a vWD-causing mutations in the vWF A2 domain are shown in blue in Fig. 4. The sites of these mutations

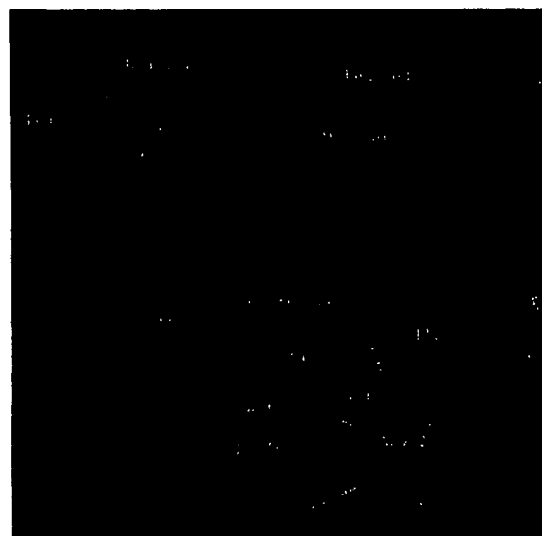


Fig. 3. Diagram of the manganese coordination site. Solvent-exposed hydrophobic residues near the manganese-binding site are shown along with the residues and two waters involved in coordinating the manganese ion. Dots indicate hydrogen bonds. Not shown is a chloride ion that occupies the sixth manganese coordination site. This figure was made with the program SBTOR (31).



FIG. 4. Sites of vWD-causing mutations. A representation of the CD11a I-domain backbone is shown in white with the manganese shown in purple. Side chains of sites of type 2a vWD-causing mutations are shown in blue and sites of type 2b vWD-causing mutations are shown in red. The mutation sites are identified in Fig. 2. The side chains shown are those of CD11a. This figure was made with the program SETOR (31).

are not localized and seem to reflect the multiple molecular mechanisms that can give rise to the type 2a vWD phenotype. In general, mutations at exposed or hydrophilic sites result in enhanced proteolysis of vWF in plasma, whereas mutations at hydrophobic sites result in impaired biosynthesis of vWF (30). Not all type 2a mutations fit this pattern, but identifying the structural location of future vWD mutations may nonetheless prove a useful tool when classifying vWD subtypes and considering possible molecular mechanisms of various forms of vWD.

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